

MOLECULAR BIOLOGY INTELLIGENCE UNIT

Mark D. Evans and Marcus S. Cooke

Oxidative Damage to Nucleic Acids

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Oxidative Damage to Nucleic Acids

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LANDES BIOSCIENCE
AUSTIN, TEXAS
U.S.A.

SPRINGER SCIENCE+BUSINESS MEDIA
NEW YORK, NEW YORK
U.S.A.

OXIDATIVE DAMAGE TO NUCLEIC ACIDS

Molecular Biology Intelligence Unit

Landes Bioscience
Springer Science+Business Media, LLC

ISBN: 978-0-387-72973-2 Printed on acid-free paper.

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Springer Science+Business Media, LLC, 233 Spring Street, New York, New York 10013, U.S.A.
<http://www.springer.com>

Please address all inquiries to the Publishers:
Landes Bioscience, 1002 West Avenue, 2nd Floor, Austin, Texas 78701, U.S.A.
Phone: 512/ 637 6050; FAX: 512/ 637 6079
<http://www.landesbioscience.com>

Printed in the United States of America.

9 8 7 6 5 4 3 2 1

Library of Congress Cataloging-in-Publication Data

Oxidative damage to nucleic acids / [edited by] Mark D. Evans, Marcus S. Cooke.

p. ; cm. -- (Molecular biology intelligence unit)

Includes bibliographical references and index.

ISBN-13: 978-0-387-72973-2 (alk. paper)

1. Nucleic acids--Oxidation. 2. DNA repair. 3. Chemical mutagenesis. I. Evans, Mark D. (Mark Dennis), 1962- II. Cooke, Marcus S. III. Series: Molecular biology intelligence unit (Unnumbered) [DNLM: 1. DNA Damage. 2. Oxidative Stress. 3. Antioxidants--therapeutic use. 4. DNA Repair Enzymes. 5. DNA Repair. QU 477 O98 2007]

QP620.O95 2007

611'.01816--dc22

2007024908

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*MDE would like to dedicate this book to his family,
relations and friends of the family—past, present and future.*

MSC would like to dedicate this book to Emily and Evie.

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PREFACE

Inspiration for this book came from a review we had written for *Bioessays*, entitled 'Factors affecting the outcome of oxidative damage to DNA'.¹ The premise was that there is a growing amount of literature examining the effects of oxidative damage to DNA, other than mutation. This included the effects of damage in transcription factor binding sites, how oxidation in CpG islands alters methylation patterns, evidence that oxidants promote microsatellite instability and even accelerate telomere attrition. We asserted that whilst, at present, on the periphery of the field of oxidative stress, compared to mutation, these factors would grow in importance, particularly as it is increasingly clear that oxidative damage to DNA has a role in diseases where mutation may not be the most significant factor.²

In this volume, we have been able to bring together many of the original authors of the articles we cited in *Bioessays*, enabling a 'first hand' description of their contribution to this most interesting area of DNA damage. One notable exception is the subject of gene-specific damage to DNA. For over ten years, this subject has received intermittent attention, largely due to the complexity of the techniques involved. This nevertheless remains an important issue and, in *Bioessays*, we provided additional focus upon both non-coding, as well as coding, regions of the genome. This work effectively rubbishes the term 'junk' DNA, if you'll forgive the pun, as the term 'junk' implies 'of no significance'. On the contrary, damage to non-coding sites can be an important event in the pathogenesis of disease.

One of the DNA oxidation products most frequently encountered throughout this book, and indeed in the scientific literature, is 8-oxo-7,8-dihydro-2'-deoxyguanosine, often abbreviated as 8-oxodG or 8-OHdG (reflective of the chemistry of this compound and its keto-enol tautomerism) and sometimes *8-oxoGuo*, *8-oxoG*. The latter two abbreviations, along with reference to the 2'-deoxynucleoside as 8-oxo-guanosine or 8-oxo-guanine are quite clearly erroneous if one is actually talking about the 2'-deoxynucleoside. While you might think us pedants for highlighting this issue, accurate distinction between the different forms of 8-oxo-guanine one can encounter (the base, the ribonucleoside and the deoxyribonucleoside) and what is actually being examined, is of prime importance when considering the provenance of such lesions measured in urine, for example. We and others have said for many years that there is no defined repair pathway for DNA that yields 8-oxo-2'-deoxyguanosine as a product, and therefore its presence in urine means something else and we also believe that its measurement has more meaning than simply as a marker of oxidation.³ This lesion has, perhaps justifiably, received the most intense research attention, but also to some extent to the detriment of the analysis of other DNA oxidation products. We and others are working to try and restore some balance.

In this book we have attempted to give a flavour of the breadth of the field, covering topics in chemistry and the formation of damage, through repair processes to placing the subject in a human health context, as well as provide a platform for some of the less considered and novel areas. Overall, we hope that this book will inform and inspire both established and young scientists alike, providing further impetus to the field, and highlight that there is more to DNA oxidation than mutation and malignancy.

1. Evans MD, Cooke MS. Factors contributing to the outcome of oxidative damage to nucleic acids. *BioEssays* 2004; 26:533-542.
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Acknowledgements

The editors gratefully acknowledge all of the authors for their sterling efforts in helping us to produce this cutting edge book.

CHAPTER 1

Oxidatively Generated Damage to Cellular DNA: Mechanistic Aspects

Jean Cadet,* Thierry Douki, Carine Badouard, Alain Favier
and Jean-Luc Ravanat

Abstract

In this chapter emphasis is placed on recent aspects of the oxidative formation of several classes of modified bases in cellular DNA that arise from the reaction of the hydroxyl radical ($^{\bullet}\text{OH}$), singlet oxygen and hypochlorous acid. Degradation compounds are detected quantitatively and specifically after suitable DNA hydrolysis into either nucleosides or bases by HPLC-tandem mass spectrometry. Thus, 6 oxidized nucleosides including: the four *cis* and *trans* diastereomers of 5,6-dihydroxy-5,6-dihydrothymidine, 5-(hydroxymethyl)-2'-deoxyuridine and 5-formyl-2'-deoxyuridine are found to be formed as the result of $^{\bullet}\text{OH}$ radical mediated oxidation of thymidine. In addition, γ -irradiation of cellular DNA was found to generate 8-oxo-7,8-dihydropurine derivatives and related formamidopyrimidine compounds resulting from $^{\bullet}\text{OH}$ radical oxidation of the guanine and adenine bases. Furthermore, singlet oxygen oxidation of guanine was found to give rise exclusively to 8-oxo-7,8-dihydro-2'-deoxyguanosine while HOCl reaction with cytosine, adenine and guanine led to the formation of 5-chlorocytosine, 8-chloroadenine and 8-chloroguanine nucleosides respectively in the DNA and RNA of human white blood cells. Interestingly, formation of these various degradation products has been rationalized in terms of existing mechanisms that were proposed previously from model studies, mostly involving free nucleosides.

Introduction

Relevant information has been gained during the last two decades on various oxidation reactions mediated by the hydroxyl radical ($^{\bullet}\text{OH}$), singlet oxygen ($^1\text{O}_2$), one-electron oxidants and hypochlorite from model studies including nucleobases, nucleosides and oligonucleotides. Thus, more than 70 modified nucleosides including diastereomeric forms and thymidine hydroperoxides have been isolated and characterized.^{1,2} In addition, relevant structural, chemical features and kinetic data on radical precursors of most of the oxidised nucleobases have become available from electron spin resonance, laser flash and pulse radiolysis analyses³. Altogether this has allowed, in conjunction with dedicated mechanistic studies, proposition of comprehensive degradation pathways for most of the oxidation reactions of purine and pyrimidine DNA bases. However, the situation is not as clear for cellular DNA since only a few oxidized 2'-deoxyribonucleosides including the four *cis* and *trans* diastereomers of 5,6-dihydroxy-5,6-dihydrothymidine (ThdGly),

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5-(hydroxymethyl)-2'-deoxyuridine (5-HmdUrd), 5-formyl-2'-deoxyuridine (5-FordUrd), 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodGuo) and 8-oxo-7,8-dihydro-2'-deoxyadenosine (8-oxodAdo), have so far been accurately detected and measured. It must be added that 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua) and 4,6-diamino-5-formamidopyrimidine (FapyAde), together with halogenated 2'-deoxyribonucleosides, have also shown to be formed in the DNA of cultured cells.⁴ These findings have been made possible by the use of appropriate methods, such as, high performance liquid chromatography (HPLC) as a suitable analytical tool that may be coupled with either the frequently used electrochemical detection technique (ECD) or the more recently available tandem mass spectrometry (MS/MS).⁵ HPLC-ECD, which was introduced 20 years ago,⁶ is a robust method whose application in the oxidation detection mode is, however, restricted to electroactive DNA lesions⁷ including 8-oxodGuo, 8-oxodAdo and 5-hydroxypyrimidine compounds. HPLC-MS/MS operating in electrospray ionization mode is more versatile and, on average, more sensitive than HPLC-ECD, allowing the measurement of numerous lesions, more often as nucleosides. It should be pointed out that previously reported gas chromatography-mass spectrometry (GC-MS) measurements of oxidized bases⁸⁻¹² have been shown to be overestimated by factors varying between 2 and 3 orders of magnitude. The main origin of the latter drawback is the occurrence of artefactual oxidation of the predominant normal nucleobases during the derivatization step, prior to the GC-MS analysis.¹³⁻¹⁶ A second source of spurious oxidation of DNA components which concerns all the assays involving DNA isolation has been identified more recently.¹⁶ This is likely to involve Fenton-type reactions during the DNA extraction step and subsequent work-up due to the presence of transition metal contaminants. Optimized DNA extraction methods involving, in particular, the use of metal chelators are now available, allowing significant reduction of the contribution of adventitious oxidation processes.^{17,18} It may be emphasized that comparative evaluation and optimization of the available HPLC and other biochemical assays aimed at measuring 8-oxodGuo in both preextracted and cellular DNA has been the subject of studies within the recent European Standards Committee on Oxidative DNA Damage (ESCODD) network.²⁰⁻²³ One of the main recommendations from these cooperative investigations, which have involved 28 laboratories, was that studies reporting levels of 8-oxodGuo greater than 5 lesions per 10⁶ normal guanines in cellular DNA are questionable and therefore should be reassessed, at least in various mammalian cells including human lymphocytes, monocytes and pig's liver. This justifies why, in the present survey devoted to mechanistic aspects of the formation of oxidatively generated base damage in the DNA of isolated cells, only the measurements of oxidized bases and nucleosides that are based on the use of HPLC-ECD and HPLC-MS/MS are reported. It may be added that appropriate physical and chemical sources of oxidizing and halogenating species were used in order to induce a significant increase above the oxidative metabolism-mediated steady-state level of investigated oxidized bases. This was achieved in an acute way under conditions where DNA repair was minimized. Hydroxyl radicals were efficiently generated by ionizing radiation, whereas endogenous photosensitizers and a thermolabile naphthalene endoperoxide were used to produce singlet oxygen (¹O₂). Chemically prepared HOCl was utilized for the halogenation of the three aminobases of DNA and RNA in SKM-1 cells. The SKM-1 leukemic cell line has been established from a patient with progression to myelomonocytic leukemia in myelodysplastic syndrome.^{23a}

Hydroxyl Radical-Mediated Oxidation of Thymine

Exposure of THP-1 human monocytes to gamma rays has been shown to induce the formation of 6 oxidized pyrimidine nucleosides that can be detected and quantified by HPLC-ECD and HPLC-MS/MS assays²⁴⁻²⁷ using, in the latter case, the accurate isotopic dilution technique, after suitable enzymic digestion of extracted DNA.²⁸ Thus 5-HmdUrd, 5-FordUrd and the four *cis* and *trans* diastereomers of ThdGly were found to be generated linearly with the dose within the dose range 90 - 450 Gy of low linear energy transfer (LET) photons from gamma rays. It may be noted that, in contrast to early data and recent HPLC-MS measurements, the yields of these oxidized nucleosides were between 29 and 97 lesions per

Table 1. Yields^a of degradation products of thymine, guanine and adenine in the DNA of THP-1 malignant human monocytes²⁶ upon exposure to γ -rays and $^{12}\text{C}^{6+}$ particles^b

Lesions	γ -Rays	$^{12}\text{C}^{6+}$ Heavy Ions
cis and trans ThdGly	97	62
5-HmdUrd	29	12
5-ForUrd	22	11
8-oxodGuo	20	10
FapyGua	39	22
8-oxodAdo	3	3
FapyAde	5	1

a) expressed in lesions per 10^9 nucleobases; b) linear energy transfer: 31.5 keV/mm.

10^9 bases Gy^{-1} , which is relatively low (Table 1). The three groups of modified nucleosides that all arose from the radiation-induced degradation of thymidine are produced upon exposure of the monocytes to high-LET $^{12}\text{C}^{6+}$ particles, however with a lower efficiency. A further decrease in the radiation chemical yield of ThdGly, 5-HmdUrd and 5-FordUrd is noted upon exposure to $^{36}\text{Ag}^{18+}$ that exhibits a higher LET value than $^{12}\text{C}^{6+}$ heavy ions.

The formation of ThdGly, 5-HmdUrd and 5-ForUrd may be mostly rationalized in terms of indirect effects of ionizing radiation that implicate the generation of $\cdot\text{OH}$. This was inferred by considering the effects of LET on the efficiency of formation of oxidized nucleosides. Interestingly, it was shown that the increase in LET led to a lower level of radiation-induced generation of the oxidized nucleosides that is concomitant with the decrease in the yield of $\cdot\text{OH}$ radical produced. The formation of the 4 diastereomers of ThdGly in cellular DNA may be accounted for by initial addition of $\cdot\text{OH}$ at C5 and, to a lesser extent, at C6 of the thymine moiety as supported by the redox titration pulse radiolysis experiments on the isolated base.^{3,29,30} In a subsequent step, fast addition of molecular oxygen takes place on the resulting reducing, 6-yl, and oxidizing, 5-yl, pyrimidine radicals in a reaction controlled by diffusion.³¹ This gives rise to related transient peroxy radicals that are subsequently reduced by the superoxide radical³² into the corresponding diastereomeric 5- and 6-hydroperoxides.^{33,34} The latter, relatively unstable, peroxidic compounds that have been fully characterized as the base and nucleoside derivatives, can be easily reduced to the corresponding alcohols, namely ThdGly,^{35,36} in a highly stereospecific way according to a $\text{S}_\text{N}2$ mechanism involving the peroxide bond.³⁷ Mechanistic insights gained from model studies including Thd and isolated DNA^{38,39} allow the depiction of the formation pathways of 5-HmdUrd and 5-FordUrd in cellular DNA. Thus $\cdot\text{OH}$ -mediated hydrogen abstraction from the methyl group of thymidine gives rise to 5-(2'-deoxyuridilyl) methyl radical which is converted into 5-(hydroperoxymethyl)-2'-deoxyuridine through the intermediacy of the related peroxy radical and subsequent reduction. Loss of a water molecule from the peroxidic function leads to the generation of 5-FordUrd whereas competitive reduction gives rises to 5-HmdUrd (Fig. 1). It is also likely that ionization processes associated with the direct effects of gamma rays would contribute, however, to a lesser extent to the oxidation reactions of the thymine moiety. The pyrimidine radical cation thus generated has been shown in model studies involving thymidine and type I photosensitizers^{40,41} to lead to the formation of ThdGly as the result of a hydration reaction giving rise to the transient 6-hydroxy-5,6-dihydrothym-5-yl radical. Competitive deprotonation of the thymine radical cation has been found to generate 5-(2'-deoxyuridilyl) methyl radical, a likely precursor of 5-HmdUrd and FordUrd in aqueous aerated solutions.^{42,43}

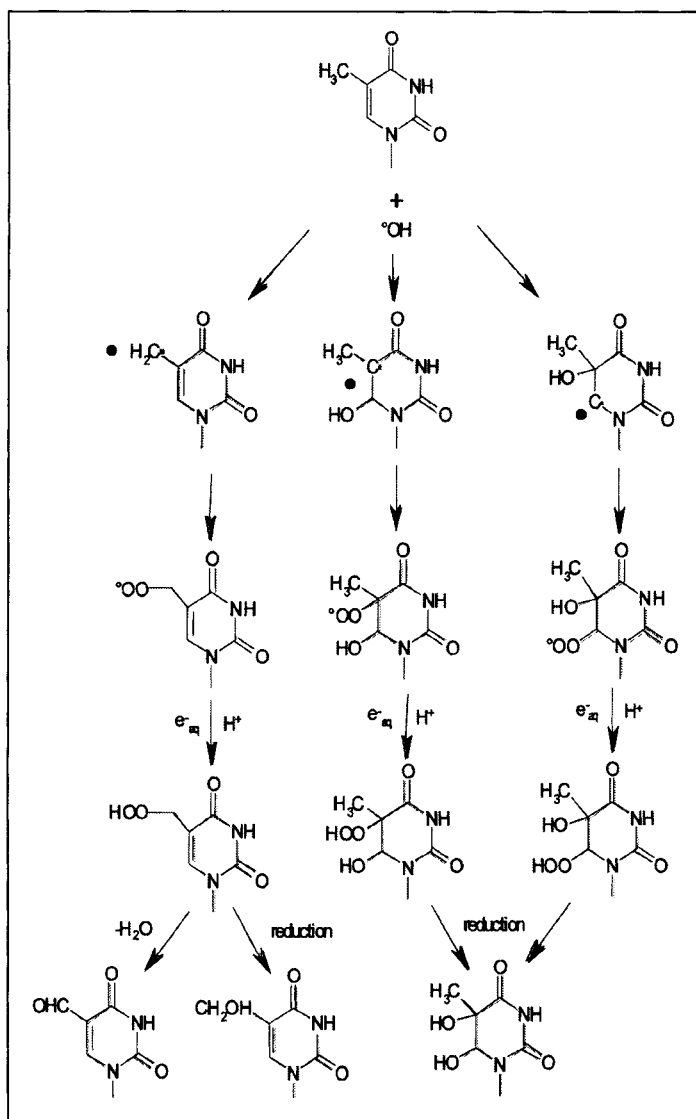


Figure 1. Hydroxyl radical-mediated oxidation of the thymine moiety in DNA.

Degradation Pathways of Purine Bases by $\cdot\text{OH}$ Radical

8-Oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodGuo), a ubiquitous, oxidatively-generated damage product of DNA that may be induced by $\cdot\text{OH}$, one-electron oxidation, peroxynitrite, $^1\text{O}_2$ ^{2,3} or as a result of intrastrand addition with thymine 5(6)-hydroxy-6(5)-hydroperoxides⁴⁴ has been found to be generated in cellular DNA upon exposure to gamma rays and high LET-heavy ions.^{26,27} This analysis was achieved after suitable enzymatic digestion of the extracted DNA and subsequent quantitative measurement by HPLC-MS/MS using the isotopic dilution method. 2,6-Diamino-4-hydroxy-6-formamidopyrimidine (FapyGua), the related opened imidazole ring compound, was also found to be efficiently generated in the DNA of irradiated human cells, using HPLC-MS/MS measurements. For this purpose, a

dedicated protocol that takes into account the instability of the *N*-glycosidic bond of formamidopyrimidines derived from purine 2'-deoxyribonucleosides in order to obtain a quantitative release of the related free base, was designed.²⁸ Interestingly, as for thymidine oxidation products, the radiation-induced formation yields of both 8-oxodGuo and FapyGua were found to decrease with the increase in the LET of the incident photon or particle (Table 1). This again is suggestive of the major implication of $^{\circ}\text{OH}$ in the molecular effects of ionizing radiation on the guanine moiety of cellular DNA. Taking into consideration the available mechanistic information that has been gained from radical oxidation studies of model systems, the following radiation-induced degradation pathway of the guanine DNA base in cells may be proposed (Fig. 2). Addition of $^{\circ}\text{OH}$ to the purine ring at C8 leads to the formation of reducing 8-hydroxy-7,8-dihydro-7-yl radical which in the presence of oxidants such as O_2 give rise to 8-oxo-7,8-dihydroguanine (8-oxoGua). A competitive reaction of the 7-yl radical is one-electron reduction that leads to the formation of FapyGua through the scission of the C8-N9 imidazole bond.⁴⁵ This was found to occur efficiently in model compounds with a high unimolecular rate ($k = 2 \times 10^5 \text{ s}^{-1}$) that was inferred from pulse radiolysis measurements.⁴⁶

It may be added that ionization processes associated with the direct effect of gamma rays are likely to contribute also to the overall radiation-induced degradation of the guanine moieties of cellular DNA. The reactions of the guanine radical cation arising from direct or indirect ionization as inferred from numerous model studies are now well documented. Hydration reactions of the guanine radical cation, produced by one-electron oxidants within double-stranded DNA, also leads to the formation of 8-hydroxy-7,8-dihydroguanyl radical^{47,48} and therefore to 8-oxoGua and FapyGua in double stranded DNA.

Exposure of human monocytes to gamma rays has been shown to generate 8-oxo-7,8-dihydro-2'-deoxyadenosine (8-oxodAdo) in the DNA and the related imidazole ring opened compound, 4,6-diamino-5-formamidopyrimidine (FapyAde) that have been measured by HPLC-MS/MS analysis. The radiation-induced formation of the two latter degradation products mimics that of the guanine base, both in isolated and cellular DNA. However, we note that the efficiency of the radiation-induced formation of each of the two adenine lesions is about 10-fold lower than that of related guanine modifications. It may be possible to

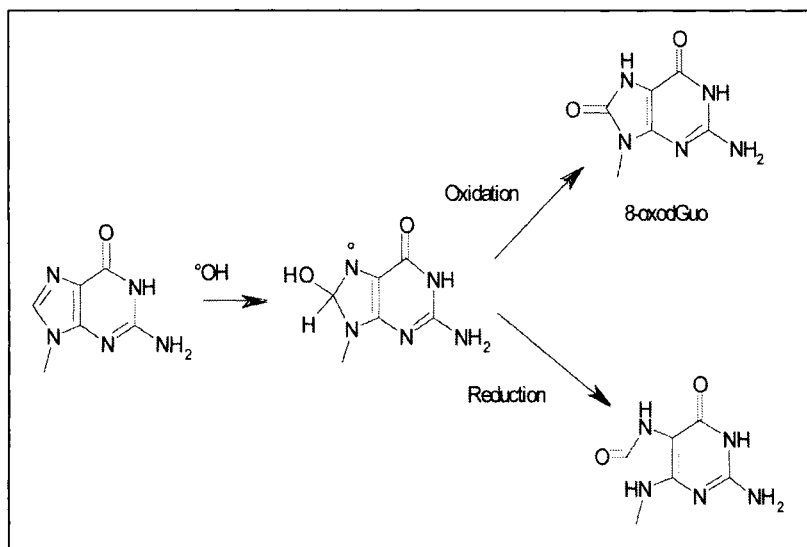


Figure 2. Hydroxyl radical-mediated oxidation of the guanine moiety in DNA.

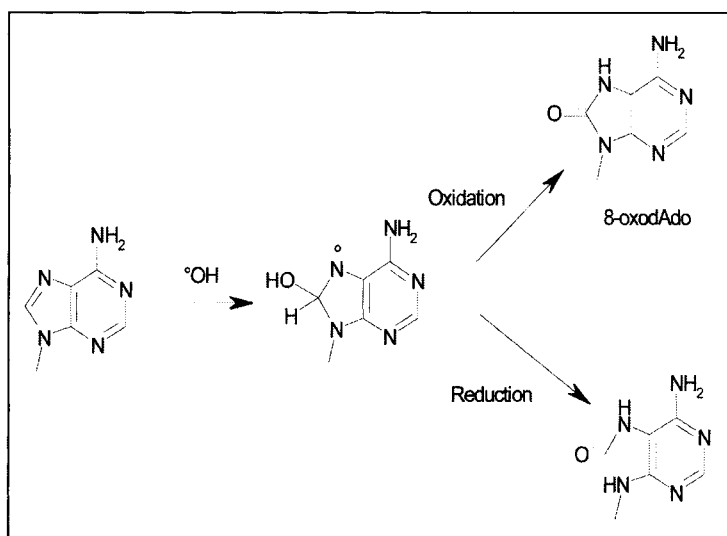


Figure 3. Hydroxyl radical-mediated oxidation of the adenine moiety in DNA.

rationalize the formation of 8-oxodAdo and FapyAde mostly in term of $^{\circ}\text{OH}$ contribution (Fig. 3). Thus, 8-hydroxy-7,8-dihydroadenyl radical formed by $^{\circ}\text{OH}$ addition at C8, is the precursor of 8-oxodA after an oxidizing step.⁴⁹ On the other hand, one-electron reduction of the latter radical⁵⁰ gives rise to FapyAde⁵¹ subsequent to scission of the 7,8-bond. Similar to what has been observed for the guanine components, hydration of the adenine radical cation generated by one-electron oxidation, has been shown to lead to the formation of 8-oxodA and FapyAde in double stranded DNA through the oxidation and reduction of the 8-hydroxy-7,8-dihydroadenyl radical precursor respectively. It may be added that the formation of 2-hydroxy-2'-deoxyadenosine (2-OHdAdo) is at best barely detectable in the DNA of γ -irradiated human monocytes as inferred from sensitive HPLC-MS/MS measurements.⁵² This contrasts with the significant yield of radiation-induced formation of 2-OHdAdo as assessed by GC-MS analysis of the DNA of γ -irradiated cells and mice.^{53,54}

Singlet Oxygen Oxidation of Guanine

$^1\text{O}_2$ in the $^1\Delta_g$ state ($E = 22.4 \text{ kcal mol}^{-1}$) may be produced in biological environments as the result of photodynamic effects provided by type II photosensitizers, or enzymatic reactions involving myeloperoxidase.^{2,40} An alternative route, that allows the generation of a clean and specific source of $^1\text{O}_2$, is provided by the use of a suitable protected naphthalene endoperoxide.⁵⁵ Thus, the release of $^1\text{O}_2$ from the thermolabile endoperoxide precursor within the cell has shown to lead to the selective oxidation of DNA guanine base moieties by producing 8-oxodG, as assessed by HPLC-MS/MS.⁵⁶ It has been further confirmed that the increase of 8-oxodG was due to singlet oxygen and not to a putative oxidative stress as inferred from labeling experiments involving a synthetically prepared [$^{18}\text{O}_2$]-endoperoxide. In addition it was found that, on the basis of the results of comet assay experiments, that $^1\text{O}_2$ released by thermal decomposition of such endoperoxides does not induce significant amounts of either direct DNA strand breaks or alkali-labile sites.⁵⁷ The formation of 8-oxodGuo in cellular DNA may be rationalized in term of initial Diels-Alder [4 + 2] cycloaddition of $^1\text{O}_2$ across the imidazole ring of the guanine moiety leading to the generation of a pair of diastereomeric 4,8-endoperoxides (Fig. 4). Support for the occurrence of the latter mechanism was provided by NMR characterization at low temperature in CD_2Cl_2 of the endoperoxide as arising from type II

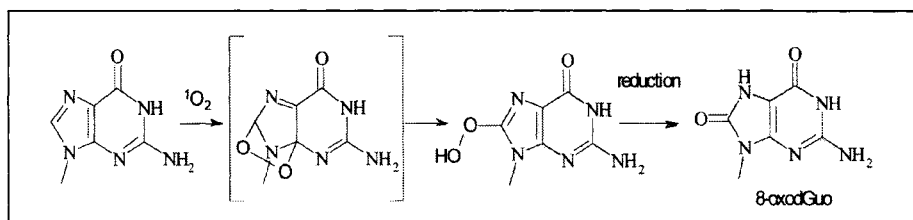


Figure 4. Singlet oxygen oxidation of the guanine moiety in DNA.

photosensitization of the 2',3',5'-O-tert-butyl dimethylsilyl derivative of 8-methylguanosine.⁵⁸ The latter intermediate has been proposed to rearrange into a linear 8-hydroperoxide.^{58,59} Further support for the latter process was provided by a recent NMR analysis of the content of the photosensitized organic solution of a 2',3',5'-O-tert-butyl dimethylsilyl derivative of 8- ^{13}C -guanosine performed at low temperature.⁶⁰ A similar situation is likely to occur in double-stranded DNA since only the formation of 8-oxodGuo has been detected.⁶¹ It has been proposed that initially generated diastereomeric 4,8-endoperoxides are able to rearrange into 8-hydroperoxy-2'-deoxyguanosine prior to reduction to 8-oxodGuo. It was also found that FapyGua is not formed, at least in detectable amounts, within isolated DNA upon exposure to a chemical source of $^1\text{O}_2$ ruling out the possibility for the latter reactive oxygen species to act by a charge transfer reaction.⁶¹ As a final remark it may be mentioned that the 4R and 4S diastereomers of spiroiminodihydantoin (dSp), which are the main $^1\text{O}_2$ oxidation products of dG,⁶²⁻⁶⁴ are not detectable in double stranded DNA.

UVA irradiation has been shown to generate 8-oxodGuo in DNA of several cell lines⁶⁵⁻⁷³ that are likely to differ in their content of endogenous photosensitizers at the origin of the observed photodynamic effects.⁷⁴ It was shown, in human monocytes, that 80 % of the formation of 8-oxodGuo in the DNA of UVA-irradiated cells was due to $^1\text{O}_2$ oxidation, as the result of type II photosensitization mechanism.²⁵ Under these conditions, the remaining 20% UVA-induced 8-oxodGuo formation was accounted for by Fenton type reactions as the result of initially generated superoxide radical and subsequent spontaneous or enzymic dismutation into H_2O_2 .²⁵

Halogenation Reactions of Nucleobases by HOCl

Hypochlorous acid (HOCl), is both a halogenating and one-electron oxidation agent⁷⁵ that is enzymatically produced by myeloperoxidase during inflammation,⁷⁶ and has been shown to induce the formation of 5-chlorocytosine (5-ClCyt), 8-chloroguanine (8-ClGua) and 8-chloroadenine (8-ClAdo) in the DNA and RNA of SKM-1 cells.⁷⁷ This was achieved using a suitable HPLC-ESI-MS/MS assay that was found to detect each of the halogenated ribo and 2'-deoxyribonucleosides in the subfemtomole range.⁷⁷ Interestingly it was shown that 5-chloro-2'-deoxycytidine (5-Cl dCyd) was generated predominantly over 8-chloro-2'-deoxyguanosine (8-Cl dGuo) and 8-chloro-2'-deoxyadenosine (8-Cl dAdo) in the DNA of SKM-1 cells upon exposure to HOCl (Fig. 5, Table 2).⁷⁷ We have noted that RNA is more susceptible than nuclear DNA to HOCl-mediated halogenation of aminobases with much higher levels of 5-chlorocytidine (5-ClCyd) and 8-chloroguanosine (8-ClGua) with respect to 8-chloroadenosine (8-ClAdo) (Table 2). It was also shown using the sensitive and specific HPLC-MS/MS assay that the level of 5-Cl dCyd was significantly more elevated in the DNA of diabetic patients with respect to healthy volunteers, suggesting the possibility of using the latter chlorinated 2'-deoxyribonucleoside as a biomarker of inflammation.⁷⁸ It may be added that the halogenation of aminobases in cellular DNA and RNA is in agreement with the results of model studies on nucleosides^{79,80} and the formation of 5-ClCyt in bacterial DNA by myeloperoxidase- H_2O_2 - Cl^- system of phagocytes.⁸¹

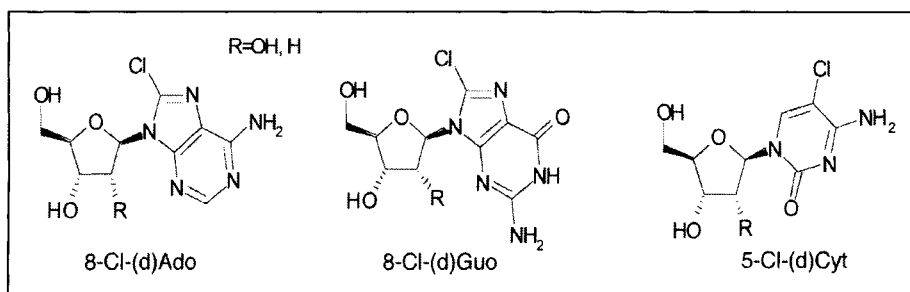


Figure 5. Chlorinated nucleosides formed in DNA and RNA.

Table 2. Yields^a of chlorinated aminobases in the DNA and RNA of SKM-1 cells upon incubation with 300 mM HOCl for 10 min⁷⁷

Chlorinated Aminobases	DNA	RNA
5-ClCyt	9.8 ± 2.3	15.8 ± 0.5
8-ClGua	2.0 ± 0.4	16.2 ± 1.8
8-ClAde	1.5 ± 0.4	0.5 ± 0.4

a) Expressed in number of lesions per 10⁶ nucleobases.

Secondary Radical Oxidation Reactions of 8-oxo-7,8-dihydroguanine

The possible occurrence of secondary oxidation reactions of 8-oxodGuo within cellular DNA⁸² has been recently highlighted by the observed accumulation of diastereomeric spiroiminodihydantoin 2'-deoxyribonucleosides (dSp) in the DNA of Nei deficient *E. coli* cells upon exposure to chromate.⁸³ The average level of dSp that was assessed by HPLC-MS in SIM mode as close to 6 dSp residues per 10⁵ guanines upon treatment of TK3D11 bacterial cells with 500 μM Cr(VI) is about 20-fold higher than in wild type cells. In both cases the level of 8-oxodGuo, that is supposed to be the precursor of dSp, was much lower than that of the latter secondary oxidation product. It was also shown previously that dSp is the main degradation product of guanine moieties in isolated DNA exposed to Cr(VI), in the presence of reducing ascorbic acid.⁸⁴ There is a growing body of evidence, from various model studies, that 8-oxodGuo, the likely initial Cr(VI)-mediated degradation product of guanine, whose oxidation potential is about 0.5 eV lower than that of dGuo⁸⁵ is a preferential target for numerous one-electron oxidizing agents and radicals.⁸⁶⁻⁹⁰ Interestingly, the *R* and *S* diastereomers of the spiroiminodihydantoin (Sp) nucleosides have been shown to be the predominant one-electron oxidation products of 8-oxodGuo and 8-oxoGuo, at neutral pH, as the result of an acyl shift of the transiently produced 5-hydroxy-8-oxo-7,8-dihydro-2'-deoxyguanosine or the related ribonucleoside derivatives, initially proposed as a relatively stable 8-oxodGuo oxidation product (Fig. 6). However it seems quite unlikely that secondary oxidation of 8-oxodGuo leading to dSp would explain the preferential formation of the latter secondary oxidation product over that of the precursor, particularly in the wild type cells.⁸³ A more likely alternative involves a more direct mechanism of dSp formation mediated by chromate that however remains to be elucidated. It would also be of interest to search for the formation of dSp using, for example, the HPLC-MS/MS technique which in the MRM mode is more accurate than the HPLC-MS/SIM method, the latter has been shown to lead to overestimated values of radiation-induced 8-oxodGuo and 8-oxodAdo in the DNA of human cells.⁹¹⁻⁹³ The lower specificity of the SIM

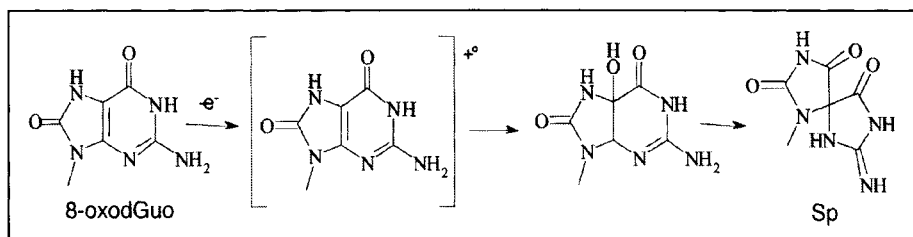


Figure 6. One-electron oxidation of 8-oxo-7,8-dihydroguanine.

detection method may explain the observation of higher values of oxidized nucleosides as the result of the presence of interfering peaks. In a more general way this should clarify the debate about the sacrificial role of 8-oxodGuo that may protect normal nucleobases within DNA against the damaging effects of one-electron oxidants^{94,95} and that has been recently questioned.⁹⁶ The question arises as to how 8-oxodGuo, which at steady state levels would not exceed a few residues per 10^6 2'-deoxyribonucleosides, could be preferentially oxidized, with respect to overwhelming nucleobase, by one-electron oxidants when hole transfer process within DNA helix does not occur in most cases at distance higher than 20 bp.

Conclusion and Perspectives

Evidence is provided in this chapter on the formation of several oxidized nucleosides and modified bases within cellular DNA upon exposure to physical and chemical oxidizing agents. This validates, at least partly, the mechanisms of degradation of nucleobases by $\cdot\text{OH}$ radical, one-electron oxidants, $^1\text{O}_2$ and HOCl . This was achieved using, for the most part, accurate and specific HPLC-MS/MS assays that have also involved optimized DNA extraction protocols in order to minimize artefactual oxidation to occur. Efforts should be made for the search of still missing lesions including cytosine oxidation products and tandem modifications such as cyclopurine nucleosides. This should benefit from, in part, at least partly of the recent availability of more sensitive HPLC-MS/MS instruments.

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CHAPTER 2

Chlorination and Nitration of DNA and Nucleic Acid Components

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Abstract

Activated phagocytes generate a complex mixture of oxidants that are believed to be crucial to bacterial cell killing. However, excessive or misplaced generation of these oxidants is known to damage host tissue. This damage is understood to be important in a number of diseases, and considerable evidence has accumulated for a link between chronic inflammation and some cancers. This is believed to occur through a variety of mechanisms, including direct damage to DNA, that can lead to mutation, and damage to enzymes, which are responsible for the synthesis and repair of DNA. In this chapter we discuss the nature and properties of oxidants [e.g., hypochlorous acid (HOCl), nitric oxide (NO) and peroxynitrite (ONOO⁻)] generated by activated phagocytes, and further reactive species [e.g., hydroxyl radicals (HO[•]), nitrogen dioxide ([•]NO₂), nitrosoperoxy carbonate (ONOOOCO₂⁻), nitrosyl chloride (NO₂Cl)] that are generated from their interactions in vivo. We focus on the reactions of these species that lead to chlorination and nitration of DNA and related nucleic acid components and examine the structural and functional consequences of these reactions. We also discuss the merits and shortcomings of using these chlorinated and nitrated DNA products as potential biomarkers of disease.

Introduction

The respiratory burst of activated phagocyte cells both in vivo and in vitro is known to generate superoxide (O₂^{•-}) from molecular oxygen via the action of NADPH oxidase enzymes.¹ A number of other cell types, including vascular smooth muscle and endothelial cells, also express functional NAD(P)H oxidases.² Subsequent dismutation of this radical, either spontaneously or via the action of superoxide dismutases, gives H₂O₂. Concurrently these cells release the heme enzyme myeloperoxidase (MPO) from intracellular granules.³ This enzyme catalyses the reaction of H₂O₂ with physiological concentrations of Cl⁻ ions to give the potent oxidant HOCl (Fig. 1), and is the only known enzymatic source of chlorinating species, under physiological conditions, in mammals.³ The pK_a of HOCl is 7.59,⁴ thus at physiological pH a mixture of both HOCl and ⁻OCl are formed; HOCl is used throughout to designate this mixture. This enzyme can also oxidise other halides (Br⁻ and I⁻), pseudo-halides (SCN⁻) and anions (e.g., NO₂⁻)⁵ as well as phenolic compounds.^{6,7} MPO shows markedly different affinities for these anions (relative specificity constants for Cl⁻, Br⁻ and SCN⁻ being 1:60:730 respectively)⁸ and as a result, at normal physiological plasma levels of these ions, approximately 45%

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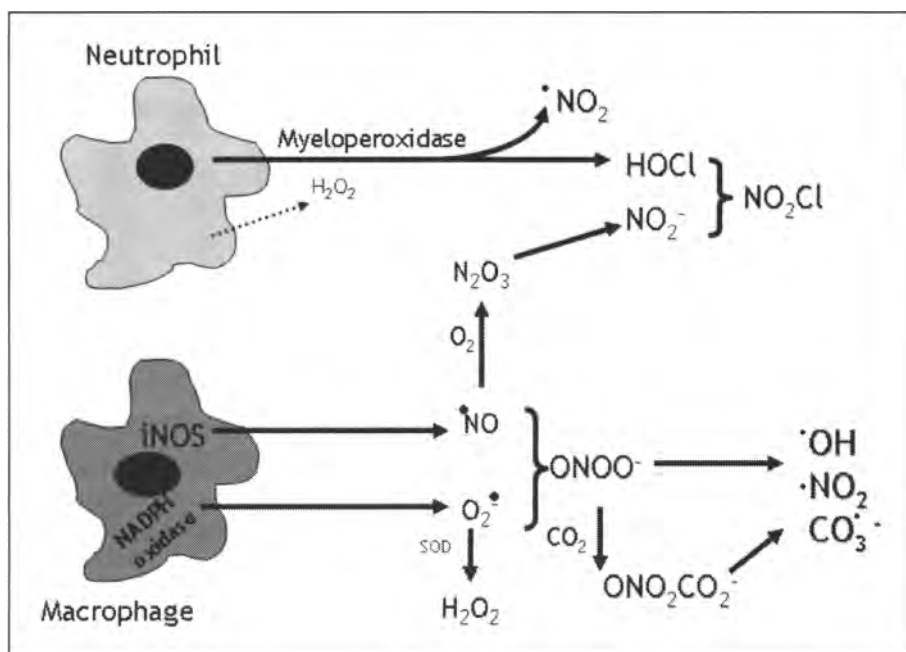


Figure 1. Generation of DNA nitrating species in vivo: Cross talk between RCS and RNS.

of the H_2O_2 consumed results in the formation of HOCl , another ca. 50% is used to oxidise SCN^- , and the remaining 5% is employed to oxidise other species.⁸ Under normal physiological conditions HOCl , and species derived from SCN^- , are therefore the major oxidants formed. The nature of the oxidants formed from SCN^- are still not well characterised,^{9,10} but it is clear that these species are less powerfully oxidising than HOCl , so attention is focussed, in this chapter, on the reactions of HOCl , and species derived therefrom.

Nitric oxide (NO) is generated from the amino acid arginine by a family of nitric oxide synthase (NOS) enzymes. These are expressed in a wide range of tissues.¹¹ Endothelial cells constitutively express endothelial NOS (eNOS), and the low levels of NO generated by this enzyme are regarded as beneficial, as this molecule is a key regulator of vascular biology.¹² Thus NO modulates vascular tone, and inhibits smooth muscle cell proliferation and platelet aggregation.¹² Neuronal cells express a second form of NOS, neuronal NOS (nNOS), which is a constitutive enzyme that plays a key role in brain function.¹¹ In contrast, inflammatory cells, such as the macrophages and T lymphocytes generate NO via an inducible form of NOS (iNOS; Fig. 1). This enzyme produces much higher concentrations of NO than eNOS and nNOS.¹³ iNOS activity is not usually detected in most normal tissues, but is a common feature at sites of inflammation where macrophage cells concentrate. Although NO is known to be able to suppress lipid and lipoprotein oxidation, by terminating radical chain reactions,¹⁴ the high levels of NO generated by iNOS have been linked with cellular damage, continued inflammation, and apoptosis as a result of the extremely rapid interaction of NO with $\text{O}_2^{\bullet -}$ to generate peroxynitrite^{15,16} and a host of other species that are commonly collectively referred to as "reactive nitrogen species" (RNS). These will be discussed in detail below.

HOCl and NO / peroxynitrite play an important role in bacterial cell killing,¹⁷⁻¹⁹ but excessive or misplaced generation of these species can cause damage to host tissue.²⁰ The oxidants react with a range of biological targets including DNA, proteins, lipids and cholesterol,^{16,21} though the rates, products and consequences of these reactions vary dramatically.

The damage induced by these oxidants is believed to be important in a number of diseases, and considerable evidence has accumulated for a link between chronic inflammation and some cancers (e.g., refs. 20,22-24). This chapter reviews current knowledge regarding damage to DNA, and related species such as RNA and nucleic acid components, induced by these oxidants. It is divided into two sections, the first dealing with chlorination and the second with nitration.

Chlorination

Formation of Reactive Intermediates

Reaction of HOCl with nucleobases, nucleosides and nucleotides appears to occur via two major processes. Firstly, reaction with the lone pair of electrons at one or more of the nitrogen atoms, where the nitrogen atom is either part of the heterocyclic ring (endocyclic) or a substituent attached to the ring (exocyclic, e.g., the free amine groups of cytosine, adenine and guanine). This results in the substitution of the hydrogen atom with chlorine, and the generation of a nitrogen-chlorine bond (often called a chloramine, where the initial reactant is an amine, or a chloramide where the initial reactant is an amide).²⁵⁻³⁰ The second type of reaction is that of (formally) Cl⁺ with the aromatic ring to give a new carbon-chlorine bond. This results in the formation of stable chlorinated products. As the nitrogen-chlorine species have been proposed to be involved in the formation of these stable products, they are discussed in this order. Furthermore, as the reactive entity in each case appears to be the nucleobase, rather than the sugar or sugar-phosphate moiety, the reactions of the nucleobases, nucleosides and nucleotides will be discussed as one group.

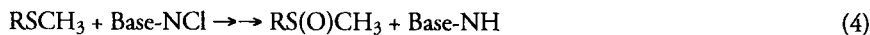
Accurate quantification of the formation of nucleobase-derived chloramines by UV spectroscopy is difficult, due to overlap between the intense UV-absorptions of the substrate with those of HOCl and its anion. The formation of N-chloro species can however, be monitored indirectly by use of their rapid reaction with thiol compounds, particularly 5-thio-2-nitrobenzoic acid (TNB), which is oxidised to the corresponding disulphide, with loss of the characteristic TNB chromophore. TNB also reacts with HOCl, so quantification of N-chloro species can only be successfully realised in the absence of residual HOCl. This is most readily achieved by use of large excesses of the substrate (which has the added advantage of precluding the formation of di-chlorinated materials), and use of time points where complete consumption of HOCl is assured—this can be ascertained either by use of reagents that react selectively with HOCl, rather than N-chloro compounds (e.g., monochlorodimedon),³¹ or by use of computer modelling of the reaction using known rate constants (see below).³² In studies where the initial yield of N-chloro compounds was assessed 5 min after addition of HOCl (0.25 mM) to 5-fold excesses of a range of substrates at 4°C, the initial yield of chloramines detected with the pyrimidine bases accounted for 95 - 100% of the initial HOCl, whereas ca. 85% and 60% conversion was observed for adenosine and guanosine respectively.²⁹ Thus, these N-chloro compounds are major initial products of HOCl-mediated oxidation of nucleobases. Studies with poly-pyrimidine compounds (poly-C, poly-U and poly-T) resulted in similar high yields of N-chloro species (90 - 95%),³⁰ with somewhat lower levels detected with poly-A and poly-G (50 - 65%).³⁰ As might be expected from these data, reaction with DNA and RNA also gave rise to high yields of these intermediates (50 - 65%).³⁰ It should be noted that with each of the materials, the exact site of N-chlorination could not be determined. In most cases, there are multiple sites at which such species could be generated.

In all of these experiments, the reaction mixtures were kept at 4°C, and were assayed as soon as practically possible after consumption of all of the HOCl. Such considerations are essential to the detection of high yields of the N-chloro compounds, as many of these are unstable species. Indeed, the somewhat lower yields of N-chloro compounds detected with the purine bases, poly-purines and DNA and RNA are believed to be due to the rapid decomposition of some of the N-chloro compounds formed on these targets prior to analysis. The stability of these compounds has been examined under a number of different conditions and it has been demonstrated that the rate of decomposition of these materials is more rapid at 22 and 37°C,

than at 4°C, and is also more rapid on exposure to UV light and metal ions.^{29,30} These compounds are also readily scavenged by reaction with compounds that contain either thioether (e.g., methionine) or free thiol groups.^{29,33} This is in accord with the weak nature of the N-Cl bond and ready chlorine transfer to compounds that possess powerful nucleophiles.³³

The degree of instability of these N-chloro compounds has been shown to vary with the structure of the substrate on which they are formed, with those formed on exocyclic amine functions being considerably more stable than those assigned to endocyclic functions (i.e., where the nitrogen is part of the heterocycle structure). Thus, a much more rapid loss of TNB-reactive material has been detected with uridine and thymidine, where only endocyclic N-chloro compounds can be formed, than with cytidine where only a slow loss of TNB reactivity was detected.²⁹ This is ascribed to the predominant formation of the exocyclic species with cytidine. With the purines, where multiple species can potentially be formed, analyses of the complex decay curves obtained suggest that both endo- and exo-cyclic N-chloro species are present. The rapid decay components are assigned to loss of the endocyclic (ring-derived) N-chloro compounds, and the slower components to decomposition of the exocyclic species.²⁹ Studies using varying excesses of substrate compared to initial HOCl have shown that the proportion of these two components in the overall decay curve varies with the substrate excess.²⁹ This has been interpreted in terms of chlorine transfer from the less stable endocyclic amine groups to give more stable, longer-lived chloramines on exocyclic amines. This interpretation is supported by the observation that chlorine transfer can occur from heterocyclic functions to other free amine functions.^{33,34} The occurrence of such transfer reactions makes the analysis of experimental decay curves difficult, if not impossible. It is likely that similar interconversion reactions occur with complex targets such as DNA and RNA, though direct evidence for this has yet to be obtained. These reactions also make analysis of the sites of damage of HOCl on such targets complex, as the site of initial N-chloro formation may be different from that detected at later time points, and different again from the site of final oxidation.

It has been shown that decomposition of these N-chloro compounds can occur via multiple mechanisms. Thus it is clear that these materials can undergo transfer reactions involving (formally) Cl⁺ to other amines (see above), thiols and thioethers.^{33,34} With the latter materials the corresponding S-chloro species have not been detected in aqueous solution as they undergo rapid subsequent reactions, resulting in the formation of disulphides (from thiols) and sulfoxides (from thioethers) (Reactions 1-4). Evidence has also been obtained for chlorine transfer to other targets, such as phenols (e.g., the amino acid tyrosine), in related studies with amino acid-derived chloramines;^{35,36} whether similar reactions occur with nucleobase-derived chloramines is not yet clear.



Related amino acid and peptide chloramines have been shown to undergo hydrolysis reactions to give carbonyl compounds, chloride ions and ammonium ions (e.g., Reactions 5-6).^{37,38} Whether similar reactions occur with nucleobase-derived species has yet to be examined. The extent of this decay pathway relative to other processes (see above and below) appears to be structure dependent. In the case of some free amino acids, this mechanism appears to predominate,^{37,38} whereas with proteins, this is only a relatively minor process.³⁹ The occurrence of similar reactions with (exocyclic) N-chloro compounds of nucleobases is of potential interest as it results in conversion of the amine function into a carbonyl function; such reactions might be expected to have effects on hydrogen-bonding and base-pairing in DNA.



The third major decomposition pathway of N-chloro compounds is via homolysis of the N-chloro bond to give a nitrogen-centred radical. This process appears to occur spontaneously (i.e., thermally) with the least stable chloramines. With more stable chloramines, appreciable rates of radical formation are only detected in the presence of UV light (which presumably results in the concurrent formation of chlorine atoms) and reducing metal ions (e.g., Cu^+ , Fe^{2+} ; Reactions 7-8).^{29,30}



The nitrogen-centred radicals formed as a result of these reactions have been detected in some cases by electron paramagnetic resonance (EPR) spin trapping using the spin trap 5,5'-dimethyl-1-pyrroline N-oxide (DMPO).^{29,30,32} The structures of the resulting radicals have been discussed in some detail, with evidence obtained for the formation of radicals centred on both exocyclic and ring (endocyclic) amine groups. In some cases the exact site of radical formation has not been determined unequivocally.^{29,30,32} These nitrogen-centred radicals are short-lived, highly reactive species and give rise, in many cases, to secondary carbon-centred radicals.^{29,30,32} The structures of some of these secondary species have been determined by EPR using the spin trap 2-methyl-2-nitrosopropane (MNP).^{29,30,32} In the majority of cases, these secondary carbon-centred radicals appear to arise from intermolecular addition of the original nitrogen-centred radical with a second molecule of parent compound to give a cross-linked species.²⁹ This conclusion is supported by the detection of similar intermolecular cross-links between (nitrogen-centred) succinimidyl radicals and some pyrimidines.²⁹ The occurrence of similar reactions in DNA has obvious implications—the formation of inter-strand cross-links would prevent DNA replication if such damage is not rapidly repaired. The extent, and significance, of these reactions remains to be determined.

Formation of Stable Products

Evidence has been obtained for the formation of stable chlorinated nucleobases on treatment of nucleobases, nucleotides, nucleosides, RNA and DNA with HOCl or a $\text{MPO} / \text{H}_2\text{O}_2 / \text{Cl}^-$ system; these involve the formation of carbon-chlorine bonds. These products include 5-chlorocytosine and 5-chloro(2'-deoxy)cytidine,^{26,27,40-43} 5-chlorouracil,^{28,44,45} 8-chloroadenine and 8-chloro(2'-deoxy)adenosine^{42,46,47} and 8-chloro(2'-deoxy)guanosine.⁴² Each of these materials is formed only in low yield (< 2%, in each case, of the HOCl consumed).⁴² A range of oxidised (hydroxylated, ring-opened) base products are also formed in these reactions, but these materials are not specific products for HOCl or the $\text{MPO} / \text{H}_2\text{O}_2 / \text{Cl}^-$ system (e.g., refs. 42,48). Similar chlorinated and hydroxylated / ring-opened products have been detected on treatment of naked DNA / RNA with HOCl.^{40,42} 8-Chloro(2'-deoxy)guanosine, 5-chloro(2'-deoxy)cytidine and 8-chloro(2'-deoxy)adenosine have been detected on stimulation of neutrophils in the presence of free nucleosides, though the yield of the last of these products was much lower than the former pair.⁴² Activated phagocytes have also been reported to generate chlorinated nucleobases in RNA and DNA.⁴⁰ More recently, 5-chlorouracil has been detected in exudate fluid isolated from a carrageenan-induced inflammation model in rats,⁴⁵ and human inflammatory tissue⁴⁹ as a result of the presence of activated phagocytes, suggesting that the above reactions are pathologically relevant.

There is some dispute over the mechanism by which these chlorinated bases are formed, with evidence presented for a role for HOCl, chloramines and molecular chlorine (e.g., refs. 41,42). At low pH values, evidence has been presented for the mediation of Cl_2 generated by MPO .⁴¹ but at higher pH values HOCl or chloramines appear to be the major chlorinating species.⁴²

Kinetics

The nucleobases are the major targets of reaction of HOCl with DNA and related compounds (as described above). Early studies of the kinetics of reaction of HOCl with nucleobases were carried out by assaying the rate of consumption of free available chlorine (FAC) using the N,N-diethyl-p-phenylene diamine/ferrous ammonium sulphate method (DPD-FAS; a standard EPA method for examining water).²⁸ Experiments with the free bases of uracil, cytosine and 5-methylcytosine resulted in the observation of mixed second order kinetics in all cases.²⁸ With uracil, the data were analysed to yield second order rate constants (Table 1). These were greater under neutral and basic conditions (pH 7 and 9) than in acidic media (pH 5),²⁸ consistent with $-OCl$ being the major attacking species. Product analysis showed quantitative formation of 5-chlorouracil.²⁸ In this study, chloramines were only detected on addition of a 4 - 5-fold molar excess of HOCl compared to uracil, which resulted in an increase (ca. 20 fold) in the observed second order rate constants for FAC consumption.²⁸ With cytosine and 5-methylcytosine, the kinetics were too complex for accurate analysis, but first order fits for the decay of FAC showed that 5-methylcytosine reacted with a rate that was ca. 60% slower than that of cytosine.²⁷ This was attributed to the presence of the methyl group blocking formation of 5-chlorocytosine and the halohydrin, 5-chloro-6-hydroxycytosine, which resulted in exocyclic chloramine formation as the major pathway.

The kinetics of HOCl reactions with mononucleotides have been followed by stopped flow methods, monitoring the small absorbance changes associated either with HOCl consumption ($\lambda \geq 290$ nm)⁴ or the loss of aromaticity of the nucleobases ($\lambda \sim 260$ nm).^{50,51} The major initial products of reaction of HOCl with nucleobases are chloramines, either on endocyclic (ring-derived $-NH$) or exocyclic amine ($-NH_2$) sites. The second order rate constants for reaction of HOCl with mononucleotides clearly fall into two groups that are dependent on the nature of the chloramine formed. The rate constants (20°C, pH 6.9) for reaction with endocyclic amines (e.g., in thymidine, uridine and guanosine monophosphates) are ca. $10^3 - 10^4$ M⁻¹ s⁻¹ (Table 1), whereas those for exocyclic amines (e.g., adenosine and cytidine monophosphates)

Table 1. Second order rate constants for the reactions of HOCl with isolated bases, mononucleotides and polymers

Target	k_2 / M ⁻¹ s ⁻¹	Product
<u>Free bases</u>		
Uracil ^a	0.28 (pH 5) 5.1 (pH 7) 5.0 (pH 9)	5-chlorouracil
<u>Mononucleotides</u>		
Uridine monophosphate ^b	5.5×10^3	Endocyclic chloramine (N-3)
Guanosine monophosphate ^c	2.1×10^4	Endocyclic chloramine (N-1)
	2.4	Exocyclic chloramine (NH ₂ at C-2)
Thymidine monophosphate ^c	4.3×10^3	Endocyclic chloramine (N-3)
3-methylthymidine ^b	No reaction	None
Adenosine monophosphate ^c	6.4	Exocyclic chloramine (NH ₂ at C-6)
Cytidine monophosphate	83 ^b , 66 ^c	Exocyclic chloramine (NH ₂ at C-4)
<u>Polymers</u>		
Poly(C) ^b	308	Exocyclic chloramines (NH ₂ at C-4)
Poly(U) ^b	1.3×10^3	Endocyclic chloramines (N-3)
DNA ^c	~ 10	Mixed chloramines

^a from reference 28; ^b at pH 6.9, 20°C, from reference 33; ^c at pH 6.9, 20°C, from reference 52.

are several orders of magnitude slower (Table 1).^{33,52} Guanosine monophosphate is the only base that contains both endo- and exo-cyclic amines, and with small excesses of HOCl over substrate, both fast and slow rates of HOCl consumption were observed, consistent with chlorination of both amine sites (Table 1).⁵² Conversely, studies with 3-*N*-methyl-thymidine monophosphate show no reactivity with HOCl, consistent with the blocking of the endocyclic amine group.⁵² The rate constant obtained for uridine monophosphate is much greater than that determined for uracil by Gould et al,²⁸ and we postulate that these slower rates may be due to secondary reactions, which result in 5-chlorouracil formation, induced by initial endocyclic chloramines, rather than by HOCl itself; this has not been confirmed.

The rate constants for reaction of HOCl with poly-C and poly-U are similar to those for the mononucleotides (Table 1),³³ indicating that polymerisation has little effect on the reactivity of the nucleobases. However, the reaction of HOCl with DNA is markedly slower (Table 1), despite the presence of both endo- and exo-cyclic amine sites; this has been attributed to protection of the amine sites by base pairing.⁵² Heat denatured DNA is chlorinated by HOCl ca. 10 times faster than native DNA consistent with this hypothesis,³³ although a much earlier report did not observe any significant difference in these rates.²⁵

Structural and Functional Consequences of Chlorination

It has been reported that treatment of DNA with HOCl results in the dissociation of the double helix as a result of the disruption of hydrogen-bonding between bases.^{33,53} Whether this is a result of N-chloro formation (i.e., replacement of N-H bonds with N-Cl) or the formation of stable carbon-chlorine products is unclear. Reaction of HOCl with plasmid DNA has been shown to result in a progressive loss of the native Type 1 form of the plasmid and an increase in the relaxed Type 2 form.³⁰ These changes could be prevented by the addition of excess methionine that removes N-chloro compounds, consistent with these species being involved in the strand breaks.³⁰ With very high (non-physiological) excesses of HOCl, complete fragmentation of the plasmid was detected. Reaction of similar plasmid samples with preformed nucleoside chloramines, but not the nucleosides themselves, also resulted in strand breaks, consistent with a key role for chloramines in these reactions. This behaviour was more rapid with ring-derived (endocyclic) chloramines than the exocyclic (free amine) species, consistent with the greater instability and reactivity of the former intermediates.³⁰ Strand breaks in plasmid DNA have also been shown to be induced by chloramines present on proteins, including those on the histone proteins, which are closely associated with DNA in the nucleus.³² These reactions are accompanied by the formation of DNA-protein cross-links, and there is strong evidence that chloramine-derived radicals play a major role in these reactions.³² The data obtained in these experiments suggest that HOCl-mediated damage to DNA within intact cells is more likely to arise via secondary reactions of chloramines formed on cellular peptides and proteins, rather than via direct reaction of HOCl with the DNA itself. Computer modelling studies support this hypothesis, with the majority of HOCl predicted to react with cellular proteins rather than DNA itself, even when only nucleosome complexes are modelled kinetically.³²

A number of halogenated bases have been shown to induce downstream cellular effects. There is evidence showing that chlorinated pyrimidine bases, upon conversion to deoxyribonucleotides, are cytotoxic, effective mutagens, clastogens, and inducers of sister-chromatid exchanges.⁵⁴ Thus, cultured mammalian cells take up and phosphorylate 5-chloro(2'-deoxy)cytidine, which can then be incorporated into genomic DNA.⁵⁵ Similarly, 5-chlorouracil can be readily converted to 5-chloro(2'-deoxy)uridine by thymidine phosphorylase, before incorporation into DNA via the action of DNA polymerase.^{54,56} 5-Chloro(2'-deoxy)uridine is a well-established thymidine analog mutagen that mispairs with guanine, causing GC-to-AT and AT-to-GC transitions.⁵⁷ 5-Chloro(2'-deoxy)uridine can also be formed from 5-chloro(2'-deoxy)cytidine via deamination mediated by cellular enzymes.⁵⁵

HOCl has been shown to induce other effects that may magnify the effects of direct DNA damage, with this oxidant shown to inhibit a number of DNA repair enzymes.⁵⁸⁻⁶⁰ It has also been shown that 5-chloro- (and 5-bromo-) cytosine can mimic 5-methylcytosine, resulting in

enhanced sequence-specific DNA-protein interactions; this could result in unintended, potentially heritable gene silencing that is postulated as a mechanism in cancer development.⁶¹

Direct evidence for a role of MPO-derived oxidants in carcinogenesis *in vivo* has been obtained from studies of the G-to-A substitution polymorphism in the promoter region of the MPO gene. Thus, it has been demonstrated in *in vitro* studies that a G-463A polymorphism decreases mRNA expression.⁶² The decrease in gene transcription caused by the variant 463A allele of MPO has been associated with a reduced risk of lung cancer.⁶³⁻⁶⁵ In contrast, the 463G allele of MPO is known to activate transcription in primary myeloid leukemia cells, resulting in an increase in MPO mRNA expression.⁶² The highest level of MPO expression is observed in acute promyelocytic leukemia, which is one of the most aggressive forms of this disease.⁶⁶

Use of Chlorinated Materials As Biomarkers of Damage

The instability of the N-chloro compounds that are the major initial products of reaction of HOCl and the complete MPO system with DNA and related materials prevents these materials being used as stable biomarkers of damage; this area has been reviewed briefly.²¹ The stable chlorinated products formed on the nucleobases as a result of carbon-chlorine bond formation are however, potential biomarkers, though it should be noted that these are minor products and hence will be difficult to quantify accurately. Indeed, previous studies have demonstrated that the artifactual generation of chlorinated DNA oxidation products can be a major problem during the analysis of biological material.⁴⁹ However, recent advances in detection methods have allowed some of these chlorinated materials to be detected reliably in human tissue⁴⁹ as a result of directly monitoring the *ex vivo* appearance of chlorinated uracil containing a ¹³C₂, ¹⁵N₁-label, from addition of [¹³C₂, ¹⁵N₁]-uracil to the biological samples prior to processing for analysis. It is likely that further technical developments will allow these materials to be used more extensively as biomarkers of DNA damage.

Nitration

Peroxynitrite (ONOO⁻) can initiate a number of DNA base modifications (nitration and deamination) as well as induce DNA strand breakage resulting in cell death by apoptotic and necrotic mechanisms. In the absence of CO₂, ONOO⁻ rapidly decomposes via a proton catalysed homolysis with $k = 1.3 \text{ s}^{-1}$ to form a nitrating and oxidising agent nitrogen dioxide (^{*}NO₂) and HO^{*}.⁶⁷ However, in the presence of CO₂, the chemistry of ONOO⁻ is further complicated by its predominant reaction with CO₂ / HCO₃⁻ to form nitrosoperoxycarbonate anion (ONOOCO₂⁻) and decomposition into ^{*}NO₂ and carbonate radicals (CO₃^{*}). Therefore consideration of the reactions of ONOO⁻ must include the reactions of all these species (ONOO⁻, ONOOH, ONOOCO₂⁻, CO₃^{*}, HO^{*}, ^{*}NO₂ and NO₂⁺) (summarised in Fig. 1).

Formation and Properties of Reactive Nitrogen Species (RNS)

Peroxynitrite (ONOO⁻)

The reaction of nitric oxide (^{*}NO) with superoxide radicals (O₂^{*}) to form peroxynitrite occurs with $k > 10^9 \text{ M}^{-1} \text{ sec}^{-1}$,¹⁵ more than 3.5 fold faster than reaction between O₂^{*} and the enzyme superoxide dismutase (SOD). Thus under conditions of increased NO production, such as chronic inflammation, ^{*}NO (synthesised from iNOS) competes with SOD for O₂^{*}, resulting in ONOO⁻ formation.⁶⁸ ONOO⁻ is an isomer of nitrate but is ca. 150 kJ mol⁻¹ greater in energy,⁶⁹ and is relatively stable in alkaline solutions. This stability is thought to be due to it being folded in the *cis* conformation.

At physiological pH, ONOO⁻ has a half-life of < 1 s due to protonation to form peroxynitrous acid, ONOOH. ONOOH is unstable at physiological pH and, in the absence of suitable substrates, rapidly isomerises to nitrate (NO₃⁻). ONOO⁻ freely penetrates phospholipid membranes ($k \text{ ca. } 8 \times 10^{-4} \text{ cm sec}^{-1}$)⁷⁰ and the cell nucleus and is able to diffuse ~100 μm before decomposition.⁷¹ During isomerisation, ONOOH forms a highly reactive intermediate, initially proposed to be HO^{*}, and ^{*}NO₂.^{68,72,73} However, HO^{*} formation from ONOOH is a subject of

considerable debate. Most EPR spin-trapping studies are consistent with $\text{HO}^\bullet$ formation,⁷⁴⁻⁷⁶ but not all.⁷⁷⁻⁷⁹ Further evidence for $\text{HO}^\bullet$ formation has been obtained from aromatic hydroxylation product studies where the substitution pattern was studied in conjunction with $\text{HO}^\bullet$ scavengers.^{80,81} However, other experiments have precluded $\text{HO}^\bullet$ formation on the basis that well-characterised $\text{HO}^\bullet$ scavengers have little or no effect on product yield.^{82,83} Initial thermodynamic calculations suggested that $\text{HO}^\bullet$ formation was unlikely, and would only contribute 10^{-6} - $10^{-4}\%$ of the overall product yield;⁶⁹ an "activated high energy" isomer of ONOOH was hypothesised to be formed instead.⁸⁴ However, recent thermodynamic calculations have concluded that $\text{HO}^\bullet$ may be formed in ca. 40% yield during ONOOH isomerisation to nitrate.⁸⁵ The peroxo bond in HOONO is weak, with a Gibbs free energy change for homolysis in aqueous solution estimated to be ca. 53 kJ mol^{-1} favouring $\text{HO}^\bullet$ and $^\bullet\text{NO}_2$ formation.⁸⁵

ONOO^- , and species derived from it, can oxidise lipids, proteins, DNA and carbohydrates^{86,87} and the addition of ONOO^- to biological fluids results in depletion of ascorbate, urate and thiols.⁸⁰ Addition of ONOO^- to cultured human or mammalian cells leads to DNA strand breakage^{88,89} and nitration of guanine residues in DNA (see below).⁸⁹⁻⁹¹ ONOO^- addition to cultured cells also leads to concomitant activation of the 'futile' DNA repair enzyme poly-(ADP)ribosyl synthetase and cell death.^{92,93} ONOO^- may also induce DNA fragmentation through the activation of the cells apoptotic machinery leading to cell death via caspase-dependent⁹⁴⁻⁹⁶ or independent processes.⁹⁷

Nitrosoperoxy carbonate (ONOOOCO_2^-)

The $\text{HCO}_3^- / \text{CO}_2$ system is one of the major pH buffering systems in the blood. The bicarbonate anion (HCO_3^-) is one of the most abundant constituents of extracellular fluids, being present at ca. 25 mM in blood plasma and 12 mM in intracellular fluids.⁹⁸ Higher levels of bicarbonate can be achieved during pathological events such as respiratory distress syndrome or ischemia reperfusion injury⁹⁹ and (probably) at inflammatory sites where ONOO^- formation occurs (reviewed in refs. 100,101). A considerable proportion of plasma and cytosolic HCO_3^- is present as carbon dioxide (CO_2) with a normal plasma concentration of ca. 1.3 mM.

ONOO^- was first noted to be unstable in carbonate buffers in 1969.¹⁰² More recent studies have shown that ONOO^- reacts with CO_2 , present in equilibrium with HCO_3^- with a rate constant of $3 - 5.8 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ at 37°C , one of the fastest reactions known for ONOO^- .^{103,104} The high plasma concentrations of $\text{CO}_2 / \text{HCO}_3^-$ make this reaction plausible *in vivo*.

The ONOOOCO_2^- adduct has been proposed to undergo either homolytic or heterolytic cleavage at the weak peroxo O-O bond to give $^\bullet\text{NO}_2$ and $\text{CO}_3^{\bullet-}$, or CO_3^{2-} and NO_2^+ ions respectively.¹⁰⁵⁻¹⁰⁷ The ratio of these two processes is disputed. $\text{CO}_3^{\bullet-}$ has been detected experimentally by EPR¹⁰⁵ and theoretical calculations and thermodynamic modelling support O-O bond homolysis.¹⁰⁸ It has been estimated that ca. 67% of the ONOO^- formed *in vivo* decomposes to NO_3^- with ca. 33% each of $^\bullet\text{NO}_2$ and $\text{CO}_3^{\bullet-}$ radicals with a cage yield of ca. 3 capable of further reactions with substrates.^{107,109,110} $^\bullet\text{NO}_2$ and $\text{CO}_3^{\bullet-}$ may also recombine to form NO_3^- and recycle CO_2 (k_2 , ca. $10^9 \text{ M}^{-1} \text{ sec}^{-1}$).¹¹¹

$\text{CO}_3^{\bullet-}$ and $^\bullet\text{NO}_2$ are powerful one-electron oxidants that oxidise appropriate electron rich species via electron transfer.¹¹² One-electron oxidation reactions of $\text{CO}_3^{\bullet-}$ are typically faster (e.g., k $4.5 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$)¹¹³ than those of $^\bullet\text{NO}_2$ (k $10^4 - 10^5 \text{ M}^{-1} \text{ s}^{-1}$). $\text{CO}_3^{\bullet-}$, but not $^\bullet\text{NO}_2$, readily oxidises guanine.¹¹⁴ In *in vitro* experiments using double-stranded oligonucleotides $\text{CO}_3^{\bullet-}$ appeared to react selectively with guanosine residues, with k $1.9 \pm 0.2 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$, giving rise to base oxidation and DNA fragmentation at such sites.^{114,115} It is possible that $\text{HO}^\bullet$ generated from ONOOH decomposition, or other pathways, also oxidises HCO_3^- to $\text{CO}_3^{\bullet-}$, which is less reactive than the $^\bullet\text{OH}$,¹¹⁶ complicating product analysis.

High, but physiological concentrations of HCO_3^- (5 - 30 mM) diminish the bactericidal and parasitocidal activity of ONOO^- ,^{98,117} inhibit ONOO^- -dependent hydroxylation and nitration of phenylalanine¹¹⁸ and benzoate⁸⁰ and inhibit methionine and GSH oxidation.^{104,119-121} However, ONOO^- -mediated nitration reactions (e.g., of guanine) are enhanced by HCO_3^- ^{90,91} at least in test tube systems.

Myeloperoxidase-Derived Nitrating Species

Myeloperoxidase (MPO) can also generate reactive nitrogen species^{122,123} through reactions with $\cdot\text{NO}$ -derived NO_2^- via 2 different mechanisms; formation of nitryl chloride (NO_2Cl) and $\cdot\text{NO}_2$. HOCl formed by MPO (see earlier) can react with $\cdot\text{NO}$ -derived nitrite (NO_2^-) forming the oxidising, nitrating and chlorinating species nitryl chloride (Reaction 9).¹²³

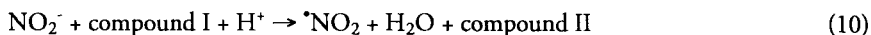


This reaction is favoured at low pH, and its second order rate constant has been estimated to be $7.4 \pm 1.3 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ (pH 7.2, 25°C).¹²⁴ Although this rate constant is relatively low, levels of HOCl and NO_2^- may be sufficiently high in vivo to permit substantial formation. Thus HOCl formation from activated neutrophils has been reported to reach $400 \mu\text{M hr}^{-1}$ ¹²⁵⁻¹²⁷ and although the NO_2^- concentration in plasma from 'healthy' volunteers is typically 0.5 - 21 μM ,^{128,129} elevated levels are present during chronic inflammation, conditions also associated with elevated HOCl levels. For example, serum NO_2^- levels in patients with rheumatoid arthritis,¹³⁰ systemic sclerosis¹³¹ and systemic lupus erythematosus¹³² are reported to be in the mM range. Additionally, NO_2^- has been used extensively for decades as a preservative and for curing meat in the food industry and NO_2^- levels in the body may represent dietary intake. Approximately 5% of ingested nitrate is reduced to nitrite by oral microflora where it enters the gastrointestinal tract and protonates to form nitrous acid (HNO_2 , pKa -3.4). This material and its anion have been proposed as an anti-microbial agent in both the gut^{133,134} and orally, where salivary levels of NO_2^- of up to 98 μM have been reported.¹³⁵ Near mM concentrations have been reported in the saliva of patients with systemic sclerosis.¹³⁶

NO_2Cl formation has been demonstrated with isolated human neutrophils after activation in the presence of NO_2^- .^{123,137} In the test tube, NO_2Cl is capable of nitrating and chlorinating phenolic compounds and inducing dimerisation (e.g., of tyrosine).¹²³ It can also increase HOCl-mediated DNA base oxidation and chlorination of cytosine⁴⁷ and xanthine.¹³⁸ It has been proposed that, at physiological pH, NO_2Cl is a more potent chlorinating agent of aromatic substrates than HOCl itself, due to the electron withdrawing character of the $-\text{NO}_2$ group that enhances the Cl^+ -character of NO_2Cl .^{123,137} This suggestion is supported by an increase in the extent of chlorination of cytosine observed on exposure of DNA to mixtures of NO_2^- and HOCl.⁴⁷

The physiological relevance of NO_2Cl as a biological nitrating agent has recently been questioned,¹³⁹ as NO_2Cl (generated from very high concentrations of NO_2^- and HOCl) has only been shown to nitrate guanine, guanosine or xanthine in the absence of cells, and does not nitrate DNA within cells. Furthermore the yields of nitrated materials detected with naked DNA are very low; for example, 8-nitroguanine and 8-nitroxanthine were generated in 0.3% and 0.71% yields respectively from DNA incubated with 580 mM NO_2^- and 300 mM HOCl.¹³⁸ These data suggest that NO_2Cl and species derived from it, may not directly effect cellular function or induce DNA nitration in vivo.

In addition to NO_2Cl formation via reactions of MPO, it has also been shown to catalyse the formation of reactive nitrogen species from $\cdot\text{NO}$ -derived NO_2^- .¹²² This reaction proceeds through the direct one-electron oxidation of NO_2^- to $\cdot\text{NO}_2$ by compound I of MPO (Reaction 10, with compound I generated from ground state MPO plus H_2O_2). This mechanism has been detected in activated neutrophils.¹²³



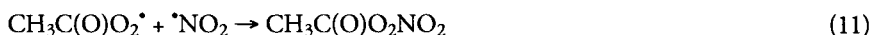
As MPO is released extracellularly, this reaction is likely to result in nitration solely, or predominantly, of extracellular components.¹³⁹ Thus MPO- H_2O_2 - NO_2^- systems (or other heme proteins)¹⁴⁰ have been shown to induce nitration solely of extracellularly-added purines rather than intracellular guanine or xanthine (Table 2).

Table 2. Generation of nitrated DNA base lesions in cell culture experiments

Cell Line / System	Base Lesion and Method of Detection	Comment	Ref.
Human lung fibroblast cells (MRC-5); nitric oxide	8-nitroguanine in cellular DNA; HPLC with UV detection at 395 nm	Cells treated with gaseous *NO (0-20 μM). Levels increased from undetectable (0 μM) to ~ 1 ng μg^{-1} DNA after 48 hrs.	158
Human keratinocytes; ONOO $^-$ and SIN-1	8-nitroguanine in cellular DNA; HPLC with UV detection. Deamination of guanine and other oxidised bases measured GC-MS. DNA strand breakage also measured.	Cells exposed to authentic ONOO $^-$ (1 mM) and SIN-1 (1 mM for 1 hr). Levels of 8-nitroguanine from 2×10^7 cells ml^{-1} increased from ~ 0.05 to ~ 8.3 pmoles μg^{-1} DNA with ONOO $^-$ and ~ 11 . Oxidation products of guanine, adenine, thymine and cytosine also observed with each treatment. ONOO $^-$ and SIN-1 induced DNA strand breakage.	89
Human neutrophils and lymphocytes; MPO / H $_2$ O $_2$ / NO $_2^-$	8-nitroguanine in cellular DNA; HPLC detection with UV detection at 387 nm. 8-hydroxyguanine also measured.	Stimulation of cells with PMA did not result in self guanine nitration even when bicarbonate was added. However, incubation of calf thymus DNA with PMA treated cells resulted in nitration of the calf thymus DNA guanine residues suggesting activated inflammatory cells produce DNA nitrating species.	161
Human lung carcinoma cells (A459); ONOO $^-$ and ONOOCO $_2^-$	8-nitroguanine in cellular RNA; HPLC with electrochemical detection.	Cells exposed to 0-120 μM ONOO $^-$ in the presence or absence of up to 25 mM bicarbonate. Untreated cells contained undetectable levels of 8-nitroguanine. The addition of 1 mM ONOO $^-$ in the absence or presence of 25 mM bicarbonate to 4×10^6 cells / 20 ml led to the formation of 27.7 ± 21 and 32.5 ± 19 μmol 8-nitroguanine / mol guanosine respectively.	159
Human neutrophils; MPO / H $_2$ O $_2$ / NO $_2^-$	8-nitroguanine and 8-nitroguanosine; HPLC with UV detection and positive ion electrospray ionisation mass spectroscopy	7×10^5 neutrophils ml^{-1} stimulated in HBSS containing PMA in the presence of 2 mM 2-deoxyguanosine and nitrite. 8-nitroguanine / 8-nitroguanosine measured in HBSS increased from 0 nM without added nitrite to ~ 120 nM with 1 mM added nitrite. Study shows MPO / nitrite system capable of nitrating extracellular and not intracellular guanosine.	160
Human neutrophils; ONOO $^-$, ONOOCO $_2^-$ and MPO / H $_2$ O $_2$ / NO $_2^-$	8-nitroxanthine; HPLC detection with UV detection at 377 nm. Confirmation with ^{13}C NMR and electrospray ionisation mass spectrometry.	2×10^6 cells ml^{-1} stimulated in HBSS containing PMA in the presence of 50 mM xanthine and 50 mM nitrite. Study shows MPO / nitrite system capable of nitrating extracellular and not intracellular xanthine.	156

Peroxyacyl Nitrates

A wide array of nitrogenated aliphatic and aromatic compounds exist in the polluted troposphere such as peroxyacyl nitrates ($\text{RC(O)O}_2\text{NO}_2$) formed as a result of the oxidation of a variety of hydrocarbons and aldehydes in the presence of nitrogen oxides (NO_x)¹⁴¹ from the combustion of petroleum and diesel, cigarette smoke and photochemical processes.¹⁴² One of the most extensively studied peroxyacyl nitrates is peroxyacetyl nitrate (PAN; $\text{CH}_3\text{C(O)ONOO}_2$). PAN in the atmosphere is formed from the reaction of $^*\text{NO}_2$ with peroxyacetyl radical formed from hydrocarbon- NO_x photo-oxidation (Reaction 11).



In the presence of excess $^*\text{NO}_2$ the rate constant of formation is $3.3 \times 10^4 \text{ s}^{-1}$ at 24°C . At 30°C PAN has a half-life of 22 min but at -10°C it is 14 days. Thus, it has been postulated to serve as a transport molecule or reservoir of atmospheric NO_x as an indicator of photochemical pollution.¹⁴³ Levels of PAN in polluted city air have been reported to reach 80 ppb¹⁴⁴ and since PAN is inhaled in polluted environments its toxicology has been extensively studied.¹⁴⁵ It is a potent lachrymatory agent with a LC_{50} of between 100 and 150 ppm and at acidic but physiologically attainable pH (<5) it is a weak point mutagen able to modify DNA bases.^{146,147} However, it was only recently that the biochemical mechanisms accounting for this were investigated.^{141,142} The addition of PAN to cultured human HL60 cells led to DNA fragmentation and apoptotic cell death¹⁴¹ as well as 8-nitroguanine formation in DNA.¹⁴² It has been postulated¹⁴¹ that PAN initiates DNA nitration and cell death through decomposition to $\text{O}_2^{\bullet-}$ and $^*\text{NO}_2$ directly (Reaction 12), or by forming ONOO^- and subsequent nitration (Reaction 13).



Although PAN is present at high concentrations in the atmosphere around polluted cities, the precise concentrations attainable within the human body are unknown, and further work is required to fully appreciate the potential role PAN may play in nitrosative and oxidative stress responses in human disease.

Mechanisms and Measurable Products of DNA Base Modification by RNS

HO^* and CO_3^* radicals react with the base, sugar and phosphate groups on DNA to cause base modification, double or single strand breakage and chromosomal aberrations.¹⁴⁸⁻¹⁵⁰ In addition, exposure of cells to oxidative stress may lead to the release of catalytic copper and iron from intracellular sites which can bind to DNA, and in the presence of ONOO^- , induce the formation of HO^* . HO^* adds rapidly to double bonds of DNA ($k > 10^9 \text{ M}^{-1} \text{ sec}^{-1}$) and efficiently abstracts hydrogen atoms ($k \text{ ca. } 10^9 \text{ M}^{-1} \text{ sec}^{-1}$),¹⁴⁸ but reacts more slowly with phosphate groups ($k < 10^7 \text{ M}^{-1} \text{ sec}^{-1}$).¹⁴⁸ As the reactions of HO^* and CO_3^* with DNA have been reviewed extensively, and $^*\text{NO}_2$ damage to the sugar-phosphate backbone is likely to generate similar intermediates to those observed with HO^* ,¹⁴⁸ only reactions that result in base nitration / nitrosation are discussed further below.

Oxidation products of the purine constituents of DNA (guanine and adenine) are the most commonly used markers of DNA oxidation. Hydroxyl radicals add to C(4), C(5) and C(8) positions of purines (to form C(4)- OH^* , C(5)- OH^* and C(8)- OH^* radical adducts respectively)¹⁴⁸ resulting in equal amounts of oxidising and reducing adduct radicals (Fig. 2).¹⁵¹ The C(4)- OH^* and C(5)- OH^* radicals of guanine dehydrate to give radicals with predominantly oxidising properties which gain an electron and proton to regenerate undamaged guanine.¹⁵² The C(8)- OH^* radicals of purines can be oxidised or reduced.¹⁵² One-electron oxidation of the C(8)- OH^* purine radical yields the 8-hydroxy derivative whereas one-electron reduction leads to the formation of imidazole ring-opened products to form diaminoformamidopyrimidine (FAPy) derivatives (Fig. 2).¹⁵³ Such a reductive process may occur, for example via intracellular thiols. Nitrated derivatives can be generated by addition of $^*\text{NO}_2$ to C(4)- and C(5)- OH^* radicals to form

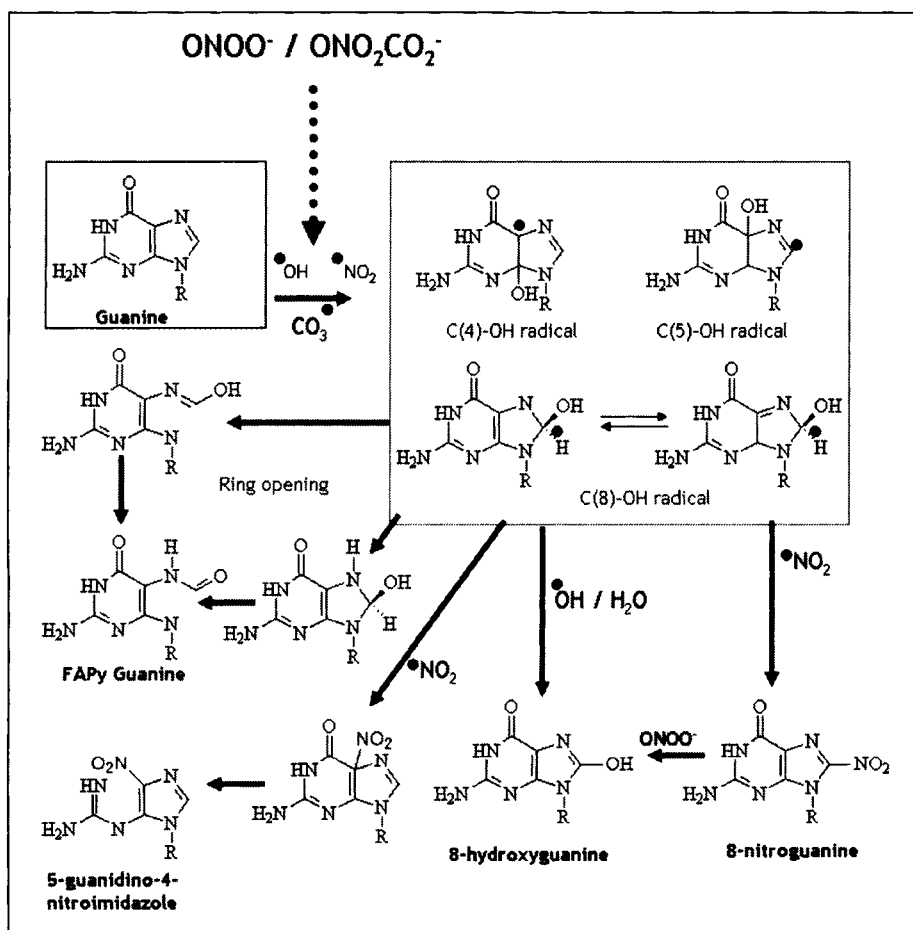


Figure 2. The formation of oxidised and nitrated guanine products by ONOO^- : The reaction of hydroxyl, nitrogen dioxide or carbonate radicals with guanine results in the formation of C(4)-OH, C(5)-OH and C(8)-OH radicals. Oxidation of C(8)-OH radicals forms 8-hydroxyguanine whereas its reduction forms the ring opened product FAPy guanine (diaminoformamidopyrimidine). Addition of nitrogen dioxide to C(8)-OH radicals results in the formation of 8-nitroguanine whereas addition to C(4)-OH and C(5)-OH radicals results in the formation of 5-guanidino-4-nitroimidazole. 8-Nitroguanine may react further with ONOO^- to form 8-hydroxyguanine.

5-guanidino-4-nitroimidazole¹⁵⁴ and the C(8)-OH[•] radicals to form 8-nitro-2'-deoxyguanosine^{90,91} and base propenal adducts.¹⁵⁵ In addition, ONOO^- deaminates guanine residues to form xanthine⁸⁹ and nitration of xanthine (8-nitroxanthine) is observed only in test tube experiments with $\text{ONOO}^- / \text{ONOOCO}_2^-$,¹⁵⁶ very high (>500 mM) concentrations of NO_2Cl ¹³⁸ and $\text{MPO-H}_2\text{O}_2\text{-NO}_2^-$ ¹⁵⁶ and has not been observed in cellular DNA. Despite this, 8-nitroguanine is widely discussed as a bio-marker for endogenous nitrative DNA damage. The formation of RNS-mediated DNA base modifications are illustrated in Figure 2.

Nitration of DNA Bases in Human Disease

8-Nitro-2'-deoxyguanosine and 8-nitro-2'-deoxyxanthosine formed in DNA are rapidly removed by depurination to yield free 8-nitroguanine and 8-nitroxanthine residues, and

mutagenic apurinic sites, with half-lives of 2^{140,157} and 4 hrs⁹¹ respectively. The 8-NO₂ adducts can be readily reduced by chemical agents *ex vivo* to give 8-NH₂ derivatives; this aids sensitive analysis as these lesions are less labile, and are stable in DNA for up to 80 hrs.^{140,157} Commercially available antibodies to 8-nitro-2'-deoxyguanosine also react with 8-nitroguanine therefore the detection of purine nitration *in vivo* reflects both 8-nitro-2'-deoxyguanosine and 8-nitroguanine formation. The addition of ONOO⁻⁸⁹ or *NO gas¹⁵⁸ to cells in culture has been shown to induce guanosine nitration in cellular DNA and RNA¹⁵⁹ but 8-nitroxanthine has yet to be found. Experiments with activated lymphocytes or neutrophils (as an *ex vivo* model of RNS generation) have not led to detectable endogenous guanine or xanthine nitration, with 8-nitroguanine and 8-nitroxanthine only detected when 2'-deoxyguanosine or xanthine were added extracellularly (summarized in Table 2).^{156,160,161}

Despite the lack of evidence for DNA base nitration in cultured cells, there is accumulating evidence for 8-nitroguanine formation in human disease. Given the strong link between inflammation, iNOS, RNS and carcinogenesis it is not surprising that studies have focused on finding 8-nitroguanine in these pathologies (summarized in Table 3). Most of these studies have employed antibodies generated *in-house*¹⁶²⁻¹⁶⁸ although commercial polyclonal and monoclonal antibodies are now available.¹⁶⁹ Analytical techniques such as high performance liquid chromatography (HPLC) and mass-spectrometry have also been used to detect 8-nitroguanine *ex vivo*.^{170,171} Clinical studies, in which DNA extracted from white blood cells from a 'healthy' population was examined, have reported 8-nitroguanine to be present at 0.02 ± 0.04 ng μg^{-1} DNA¹⁷⁰ and 0.08 ± 0.03 ng μg^{-1} DNA.¹⁷¹ Cigarette smoking elevated 8-nitroguanine levels to 1.43 ± 0.79 ng μg^{-1} DNA whereas DNA from heavy smokers with lung cancer contained 3.62 ± 1.38 ng μg^{-1} DNA.¹⁷⁰ In contrast, patients with gouty arthritis had only 0.59 ± 0.09 ng μg^{-1} DNA.¹⁷¹ Cigarette smoke contains a variety of RNS so DNA nitration *in vivo* may proceed through both iNOS-dependent and independent mechanisms.

In the cancer studies performed to date, 8-nitroguanine immunoreactivity colocalised with cells expressing the p53 protein,¹⁶⁵ proliferating cell nuclear antigen (PCNA, a marker for cell proliferation),^{165,166} heme oxygenase-1 protein expression, iron deposition¹⁶² and hypoxia-inducible factor-1 α .¹⁶⁴ 8-Nitroguanine immunoreactivity was also associated with increasing tumour invasiveness suggesting that 8-nitroguanine may be a reliable predictor for cancer progression. Interestingly, 8-nitroguanine was found in the cytosol of cells as well as the nucleus consistent with either RNA nitration, or nuclear excision repair.^{165,167}

Consequences of DNA Nitration

In addition to DNA base nitration, RNS may be associated with increased DNA damage through inhibition of DNA repair pathways,¹⁷² including those involving the enzymes Fpg,¹⁷³ DNA ligase,¹⁷⁴ 8-oxoguanine-DNA glycosylase¹⁷⁵ and O⁶-methylguanine-DNA methyltransferase.¹⁷⁶ *NO and ONOO⁻ also inhibit repair enzymes involved in nucleotide excision repair.¹⁷⁷ Accumulation of DNA damage induced by RNS has been reported to either activate,¹⁷⁸ or inhibit, the DNA repair protein p53.¹⁷⁹ Since p53 is involved in initiating repair of damaged DNA, it is tempting to speculate that RNS-induced DNA nitration and p53 inactivation may increase the survival of precancerous cells leading to carcinogenesis. Interestingly, the p53 protein has been observed to be nitrated in gliomas¹⁸⁰ and 8-nitroguanine was recently shown to colocalise with p53 and PCNA in a mouse model of inflammatory bowel disease¹⁶⁵ consistent with 8-nitroguanine being a reliable bio-marker for carcinogenesis.

RNS-Mediated Decomposition of Oxidised DNA Base Lesions

Although 8-hydroxyguanine is often the most abundant, and most studied DNA lesion, exposure of isolated DNA or cells to ONOO⁻ has given rise to conflicting data with regard to the presence and level of this lesion. It has only been detected when very low ONOO⁻ concentrations have been employed, with this material being reported to be absent with higher levels of ONOO⁻. This is explicable in terms of the ready oxidation of 8-hydroxyguanine, a fact

Table 3. Detection of nitrated DNA base lesions in vivo / ex vivo: Links to cancer development and chronic inflammation

Observation	Method of Detection	Comment	Ref.
Liver of hamsters infected with <i>Opisthorchis viverrini</i>	Immunohistochemistry with commercial 8-nitroguanine polyclonal antibody (Dojindo)	<i>Opisthorchis viverrini</i> infection is a high risk factor for cholangiocarcinoma.	169
Potential role in the development and progression of and hepatic cancer.		Extent of 8-nitroguanine immunostaining correlated with a rise in plasma nitrite / nitrate and 8-hydroxyguanine. 8-nitroguanine immunoreactivity observed in the cytoplasm and nucleus of inflammatory cells and epithelial cells lining the bile duct.	
	Immunohistochemistry with polyclonal 8-nitroguanine antibody developed by the authors	Repeated infection led to sustained elevation of iNOS protein and accumulation of 8-nitroguanine in bile duct epithelial cells. 8-Nitroguanine levels positively correlated with iNOS protein and proliferating cell nuclear antigen.	163
		Positive correlation of 8-nitroguanine levels with heme oxygenase-1 protein expression and iron deposition in bile duct epithelial cells, Kupfer cells and sinusoidal cells in infected liver tissue. Time course study revealed elevations of 8-nitroguanine were parallel to rises in iNOS protein synthesis.	162
		Levels of 8-nitroguanine, iNOS and hypoxia-inducible factor-1 α colocalised in cancerous tissues and were associated with increasing tumour invasiveness.	164
Mouse model of inflammatory bowel disease	Immunohistochemistry with polyclonal 8-nitroguanine antibody developed by the authors	Levels of 8-nitroguanine colocalised with iNOS, proliferating cell nuclear antigen and p53 proteins as well as 8-hydroxydeoxyguanosine in the nucleus in colonic epithelial cells.	165
Potential role in colonic cancer			
Gastritis with <i>Helicobacter pylori</i> infection. Human study	Immunohistochemistry with polyclonal 8-nitroguanine antibody developed by the authors	8-nitroguanine immunoreactivity colocalised with 8-hydroxyguanine in corpus and antrum of gastric gland epithelium of <i>H. pylori</i> infected gastritis patients. Levels positively correlated with proliferation cell nuclear antigen and positively correlated to the extent of <i>H. pylori</i> infection.	166
Possible role in gastritis and gastric cancer			

continued on next page

Table 3. Continued

Observation	Method of Detection	Comment	Ref.
Tobacco smoking / tobacco smoking and lung cancer patients. Human study	High performance liquid chromatography with electrochemical detection with confirmation by electrospray mass spectrometry	8-nitroguanine isolated from peripheral lymphocytes of smokers where levels increased with tobacco smoke intake and positively correlated with serum levels of nitrite. Levels of 8-nitroguanine reported as: Nonsmokers, 0.02 ± 0.04 ng μg^{-1} DNA; light smokers 0.9 ± 1.0 ng μg^{-1} DNA; medium smokers, 1.23 ± 1.14 ng μg^{-1} DNA; heavy smokers, 1.43 ± 0.79 ng μg^{-1} DNA; heavy smokers with lung cancer, 3.62 ± 1.38 ng μg^{-1} DNA.	170
Implications for cigarette smoking and lung cancer		8-nitroguanine observed in lung homogenates and peripheral lymphocytes of tobacco-exposed rats. Levels increased with increasing smoke exposure.	
Viral pneumonia in mice	Immunohistochemistry with polyclonal 8-nitroguanine antibody developed by the authors	Viral pneumonia was induced by inhalation of influenza virus A/Kumamoto/5/67 (H2N2) and Sendai virus Z strain suspensions. 8-nitroguanine immunostaining observed in the cytosol of bronchial and bronchiolar epithelial cells where it colocalised with iNOS protein. Positive correlation observed with levels of 3-nitrotyrosine (protein marker of RNS-mediated protein nitration) in the lungs of infected mice. 8-Nitroguanine immunostaining was not observed in iNOS ^{-/-} mice.	167
Role in viral infection as a diagnostic marker		Link to viral infection.	
Hepatitis C infection and nonalcoholic fatty liver. Human study	Immunohistochemistry with polyclonal 8-nitroguanine antibody developed by the authors	8-Nitroguanine observed in hepatocytes. Levels positively correlated with increasing degree of liver inflammation but not extent of fibrosis and decreased after patients received interferon-gamma treatment.	168
Inflammatory gouty arthritis. Human study	HPLC with electrochemical detection of 8-nitroguanine in total white blood cells.	White blood cell levels of 8-nitroguanine correlated positively with levels of C-reactive protein, plasma levels of nitrite / nitrate, uric acid and white blood cell count.	171
Role in chronic joint inflammation		Levels of 8-nitroguanine reported as: non inflammatory controls, 0.08 ± 0.03 ng μg^{-1} DNA; gouty arthritis with normal leucocyte count, 0.34 ± 0.13 ng μg^{-1} DNA; active gouty arthritis with leucocytosis 0.59 ± 0.09 ng μg^{-1} DNA.	

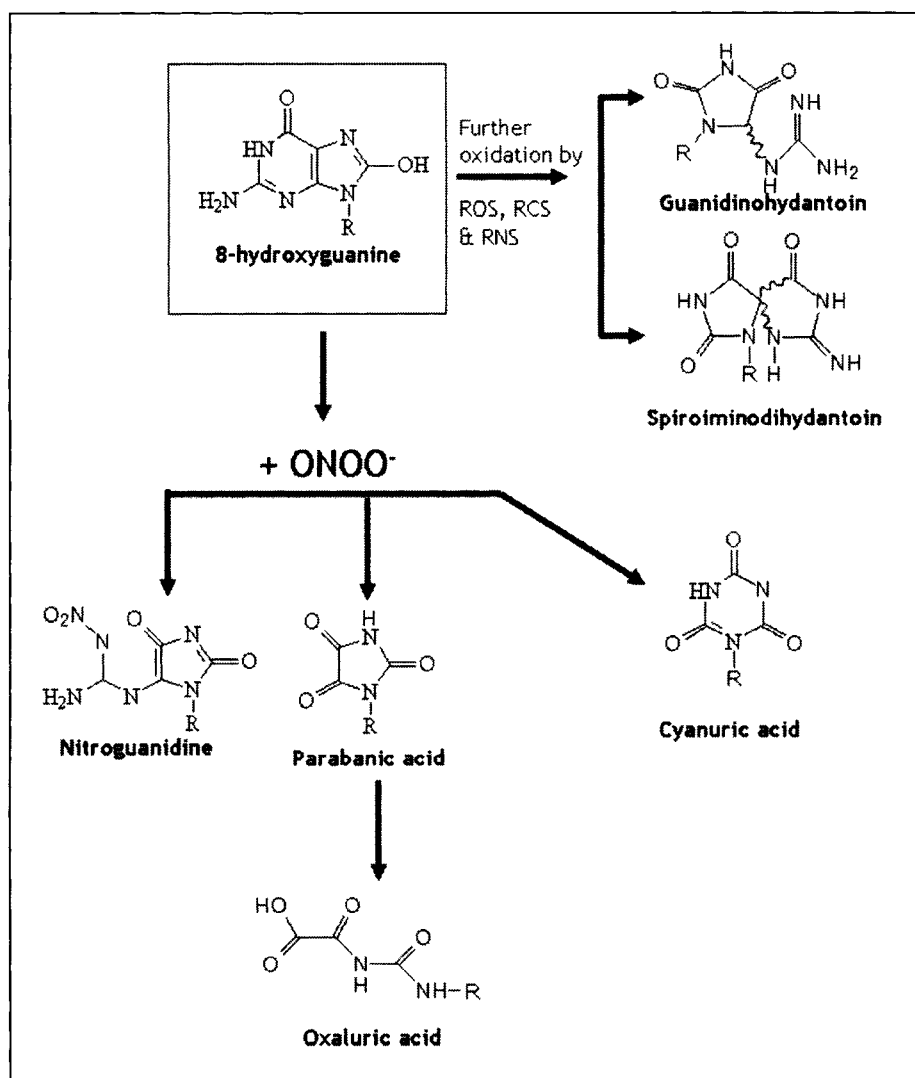


Figure 3. ONOO⁻-mediated degradation of 8-hydroxyguanine. 8-hydroxyguanine may be further oxidised to produce guanidinohydantoin, spiroiminodihydantoin and N-nitro-N-[1-(2,3,5-tri-O-acetyl-D-erythro-pentofuranosyl)-2,4-dioxoimidazolidin-5-ylidene]guanidine (nitroguanidine).

exploited in the detection of this material by electrochemical oxidation. Since ONOO⁻ is a powerful one-electron oxidant, as are many of the radicals formed from it (HO[•], E 1.90 V; CO₃^{•-}, E 1.5 V; [•]NO₂, E 1.04 V),^{181,182} these species are all thermodynamically capable of oxidising 8-hydroxyguanine (E 0.58 V)¹⁸³ to give a radical-cation.^{152,184} ONOO⁻-derived [•]NO₂ readily combines with 8-hydroxyguanine radicals in oligonucleotides, and presumably DNA, with rate constants $k > 4.2 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$.¹⁸⁵ Addition of ONOO⁻ to DNA containing hydroxylated bases therefore leads to the rapid and extensive loss of 8-hydroxyguanine as well as other lesions.⁴⁷ Reaction of ONOO⁻ with 8-hydroxyguanine results in multiple intermediates,¹⁸⁶⁻¹⁸⁸ with the formation of additional ring nitrated products such as 5-guanidino-4-nitroimidazole

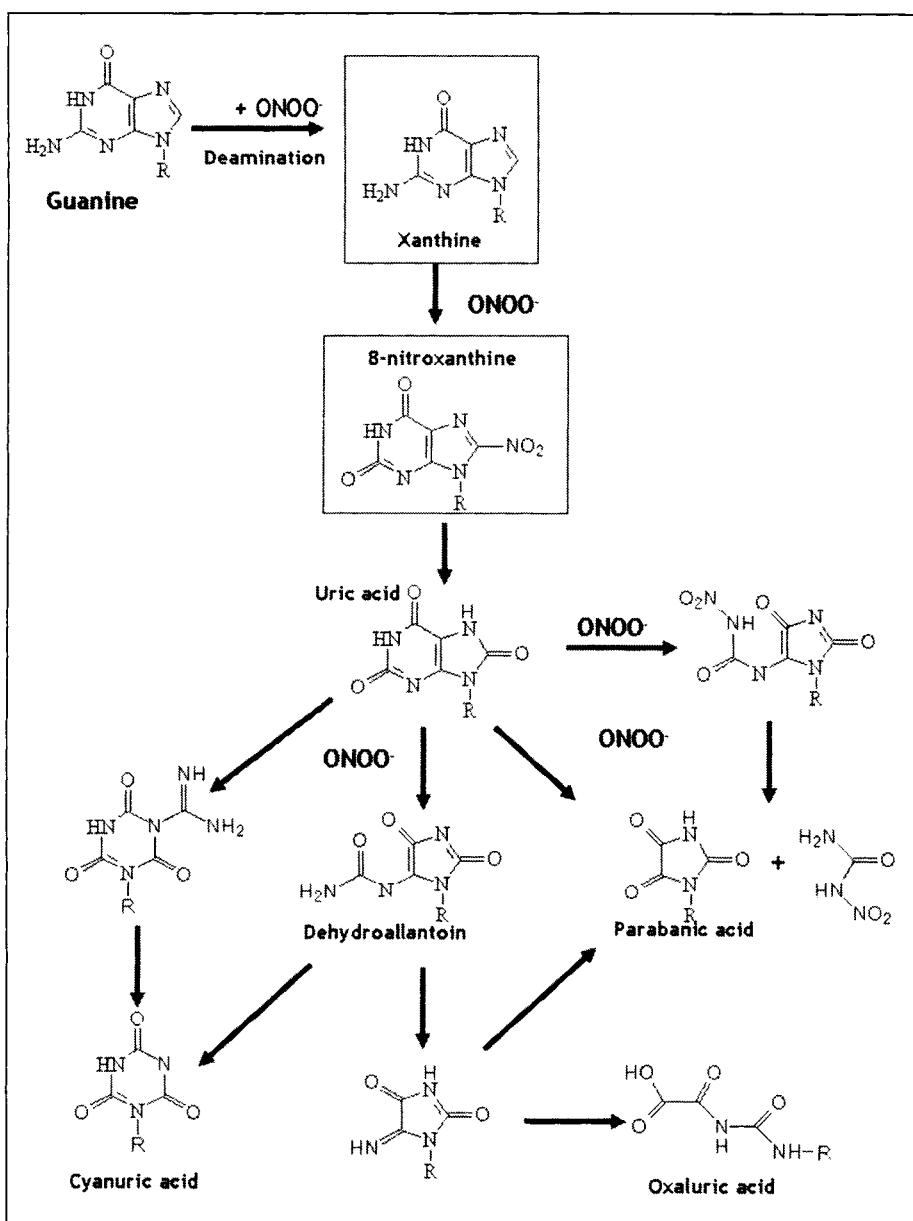


Figure 4. ONOO^- -mediated degradation of nitropurines. Modified from reference 189.

and the *N*-nitration product, nitroguanine (Fig. 3). Similarly, 8-nitroxanthine reacts with ONOO^- to form further products (Fig. 4) and 8-nitroguanine may also further react with ONOO^- to generate 8-hydroxyguanine. However, in contrast to 8-nitroguanine and 8-hydroxyguanine, these additional products have only been detected in vitro experiments using isolated 8-hydroxyguanine or calf thymus DNA, and not in cell cultures or in vivo. Therefore the measurement of 8-hydroxyguanine may not represent a true extent of DNA base oxidation in vivo.⁴⁷

Summary and Conclusions

Considerable advances have been made over the last few years in determining the kinetics and reactions of chlorinating and nitrating species with isolated DNA and its components. It is now clear that a range of different oxidants can induce both direct and indirect modification of these materials, and that some of the products of these reactions can be used as quantitative markers of DNA damage. The situation with intact cells and animals, is however, less clear-cut and considerable further work needs to be carried out to determine whether similar reactions and processes occur in vivo. Only when such studies have been completed can the true role of these species in mutation and carcinogenesis be fully comprehended.

Acknowledgements

The authors thank the Australian Research Council and the National Health and Medical Research Council (C.L. Hawkins, D.I. Pattison and M.J. Davies) and the National Medical Research Council and NUS Office of Life Sciences, Republic of Singapore (M. Whiteman) for their continued and generous research support.

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CHAPTER 3

Prevention of the Mutagenicity and Cytotoxicity of Oxidized Purine Nucleotides

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Abstract

Damage to nucleic acids is particularly hazardous because the genetic information in genomic DNA, such as nuclear and mitochondrial DNA, can be altered. Damage accumulated in cellular DNAs often initiates programmed cell death, as well as mutagenesis. The former may cause degenerative diseases, and the latter may result in neoplasia and hereditary diseases. The accumulation of oxidative damage in cellular DNA or RNA is a result of the incorporation of oxidized nucleotides generated in nucleotide pools, as well as a result of their direct oxidation. Recent progress in studies of the sanitization of nucleotide pools, in addition to DNA repair, have revealed the significance of the oxidation of free nucleotides to be unexpectedly large, in comparison to the direct oxidation of DNA.

Introduction

Amongst the various types of oxidative damage to cellular macromolecules, damage to nucleic acids is particularly hazardous because the genetic information present in genomic DNA, nuclear and mitochondrial DNA, can be altered. The damage accumulated in cellular DNA often results not only in mutagenesis but also in programmed cell death, and the former may initiate carcinogenesis in somatic cells and mutations fixed in germ lines cause genetic polymorphism or result in hereditary diseases with a malfunction of the gene(s), while the latter often causes degenerative diseases.¹ There are two alternative pathways for the accumulation of oxidized bases in cellular DNA or RNA: one is a result of the incorporation of oxidized nucleotides generated in nucleotide pools; while the other is a result of the direct oxidation of bases. Recent progress in studies of the sanitization of nucleotide pools, as well as DNA repair, has revealed that the impact of oxidation of free nucleotides is unexpectedly large, in comparison to the direct oxidation of DNA.^{2,3} In this chapter, we focus on the oxidation of purine nucleotides and review the biological consequences that have been revealed in either studies with mutant cells or in animals lacking such sanitizing enzymes.

Oxidation of Free Nucleotides and Their Mutability

Kasai and Nishimura⁴ reported that among the guanine residues in various forms of nucleic acids such as deoxyguanosine (dG), poly G, poly(dG-dC):poly(dG-dC), denatured or native calf thymus DNA, the C-8 position of dG is the most effectively oxidized by ascorbic acid in the

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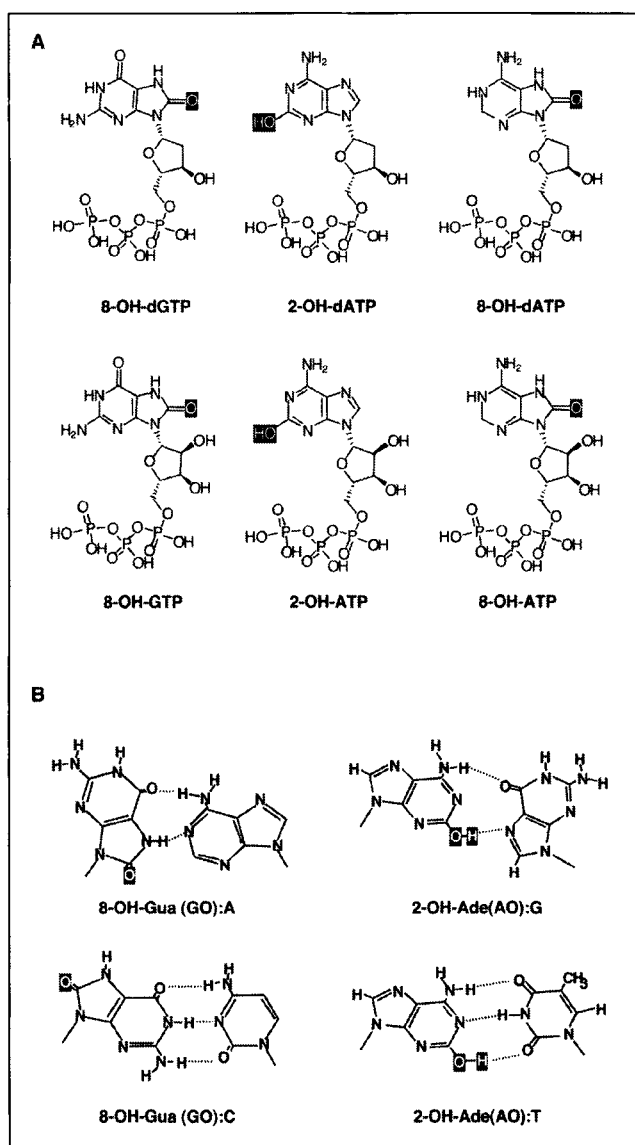


Figure 1. Oxidized purine nucleoside triphosphates and their base pairing. A) The structure of oxidized purine nucleoside triphosphates. The chemical structures were drawn using CS ChemDraw (Cambridge Soft, Cambridge MA). The oxygen or hydroxy group acquired from ROS is shown in black box. B) 8-OH-Gua (GO) and 2-OH-Ade (AO) can pair with adenine (A) or guanine (G) in template DNA, respectively, during DNA replication.⁶⁹

presence of O_2 or H_2O_2 (Fig. 1A). They also showed that the reaction was catalyzed by Fe^{2+} -EDTA ($FeSO_4$, EDTA). Additionally, Mo et al⁵ reported that the incubation of dGTP with H_2O_2 and ascorbic acid results in a conversion of up to 10% into 8-hydroxy-2'-deoxyguanosine triphosphate (8-OH-dGTP). Later, Kamiya and Kasai⁶ demonstrated that treatment of dGTP with Fe^{2+} -EDTA generates 8 to 9 times more 8-hydroxy-7,8-dihydroguanine (8-OH-Gua) residues in the nucleotide dGTP than in DNA. Interestingly, the C-8 position of dATP is not attacked in the

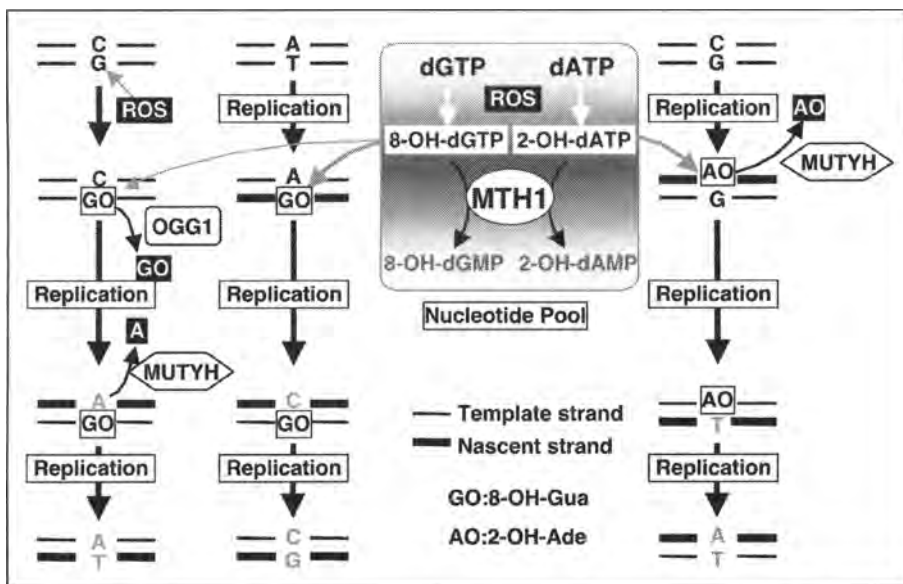


Figure 2. Mutagenesis caused by the oxidation of nucleic acids and error avoiding mechanisms in mammals. 8-OH-Gua accumulates in DNA, as a result of the incorporation of 8-OH-dGTP from the nucleotide pools or because of direct oxidation of DNA, increases the occurrence of A:T to C:G or G:C to T:A transversion mutation, while 2-OH-Ade is mainly derived from the incorporation of 2-OH-dATP from nucleotide pools. The accumulation of 8-OH-Gua or 2-OH-Ade in DNA is minimized by the coordinated actions of MTH1 (oxidized purine nucleoside triphosphatase), OGG1 (8-OH-Gua DNA glycosylase) and MUTYH (Adenine/2-OH-Ade DNA glycosylase).⁶⁹ GO: 8-OH-Gua, AO: 2-OH-Ade. Bold lines: Nascent strands of DNA.

treatment, instead, the C-2 position of dATP was effectively oxidized, thus yielding 2-hydroxy-2'-deoxyadenosine (2-OH-dATP) (Fig. 1A). However, treatment with Fe^{2+} -EDTA did generate 2-hydroxy-adenine (2-OH-Ade) residues in DNA as little as 1.5% of the level of 2-OH-Ade residues that are formed from dATP. To obtain 8-OH-dATP (Fig. 1A), γ -irradiation was applied to dATP in solution.⁷ As a result, free nucleotides are more susceptible to oxidation by reactive oxygen species (ROS) than DNA, however, it is very difficult to detect such oxidized nucleotides in vivo,⁸ probably because the dNTP precursors are newly synthesized just prior to DNA replication.^{9,10}

It has been established that 8-OH-dGTP and 2-OH-dATP are frequently misinserted opposite an incorrect base in the template DNA by various replicative DNA polymerases, from bacteria to humans (Fig. 1B).¹¹⁻¹⁴ Furthermore, Inoue et al¹⁵ showed that these oxidized nucleotides indeed increased certain mutations when they were introduced into *E. coli* cells. As summarized in Figure 2, 8-OH-dGTP is misinserted opposite adenine as well as cytosine in the template DNA, thus causing mainly an A:T to C:G transversion mutation after two rounds of replication. 2-OH-dATP tends to mostly be misinserted opposite guanine, thus inducing mainly G:C to T:A transversion mutation.

Removal of Oxidized Nucleotides by Sanitization of Nucleotide Pools

E. coli mutT mutants exhibit the strongest mutator phenotype among all known *E. coli* mutator mutants, and the spontaneous occurrence of A:T to C:G transversion mutation increases 1000-fold in comparison to wild type.¹⁶ Maki and Sekiguchi¹¹ demonstrated that

MutT protein hydrolyzes 8-OH-dGTP to 8-OH-dGMP and pyrophosphate, thus sanitizing nucleotide pools. MutT protein also efficiently hydrolyzes 8-OH-GTP, and *mutT* mutants accumulate 8-OH-Gua in mRNA which also results in the production of mutant proteins.¹⁷ *E. coli* has a back-up enzyme for MutT, namely RibA (GTP cyclohydrolase II) and it partially suppresses the *mutT* mutator phenotype by hydrolyzing 8-OH-dGTP.¹⁸ The *E. coli* Orf135 protein was recently reported to be able to hydrolyze 2-OH-dATP,¹⁹ while Orf17 (NtpA) protein hydrolyzes 8-OH-dATP and 8-OH-dADP as well as dATP/dADP.²⁰ Orf135 mutants exhibit a 2-fold increase in the spontaneous occurrence of A:T to C:G transversion, and the introduction of 2-OH-dATP, but not 8-OH-dGTP or other nucleotides, into Orf135 mutants, specifically increases the mutation frequency in comparison to wild type.²¹ MutT, Orf135 and Orf17 proteins share only the phosphohydrolase module or MutT signature, corresponding to the 23 residues from Gly37 to Gly59 of *E. coli* MutT, which constitute the active center hydrolyzing phosphodiester bonds of the substrates (Fig. 3).²²⁻²⁴

We have previously identified human homolog of the MutT protein and designated it as MTH1 (MutT homolog-1).^{5,25,26} We reported that MTH1, but not MutT, efficiently hydrolyzes 2 forms of oxidized dATP, 2-OH-dATP and 8-OH-dATP, as well as 8-OH-dGTP,⁷ while MTH1 also hydrolyzes 2-OH-ATP, 8-OH-GTP and 8-OH-ATP.²⁷ Among them, MTH1 has the highest affinity to 2-OH-ATP ($K_m = 4.3 \mu\text{M}$), while the highest catalytic efficiency was observed for 2-OH-dATP ($k_{cat}/K_m = 1.68 \text{ s}^{-1}\mu\text{M}^{-1}$) (Fig. 2). We recently determined the solution structure of MTH1 by multi-dimensional heteronuclear NMR spectroscopy (Fig. 4A).²⁸ The protein adopts a highly similar folding pattern to *E. coli* MutT,²³ despite the low sequence similarity outside the conserved phosphohydrolase module.^{29,30} The substrate binding pockets are dissimilar to each other, which may account for the different substrate specificities observed for the two enzymes. Based on the arrangement of the pocket-forming residues, combined with the mutagenesis data, we generated models for the substrate recognition of MTH1, in which Asn-33 and Asp-119 play pivotal roles in discriminating the oxidized

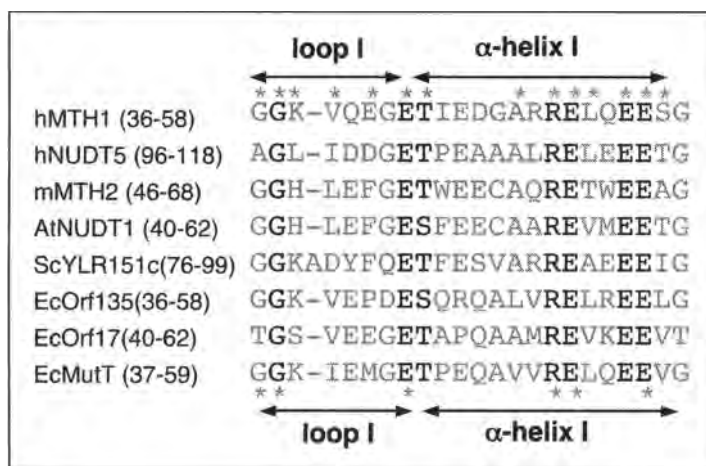


Figure 3. Phosphohydrolase module conserved among various enzymes which hydrolyze oxidized purine nucleoside triphosphates. The 23 residue modules from human MTH1 (hMTH1), hNUDT5, mouse MTH2 (mMTH2), *A. thaliana* NUDT1 (AtNUDT1), *S. cerevisiae* YLR151c (ScYLR151c), *E. coli* Orf135 (EcOrf135), Orf17 (EcOrf17), and MutT (EcMutT) proteins are aligned. Residues 36-43 in hMTH1 and residues 37 to 45 in *E. coli* MutT constitute loop I, residues 44-58 in hMTH1 and residues 46 to 59 in MutT constitute α -helix I. Conserved amino acid residues are shown in black. The residues with an asterisk in hMTH1 and MutT indicate those that could not be replaced by any other residue without losing their function.^{22,30}

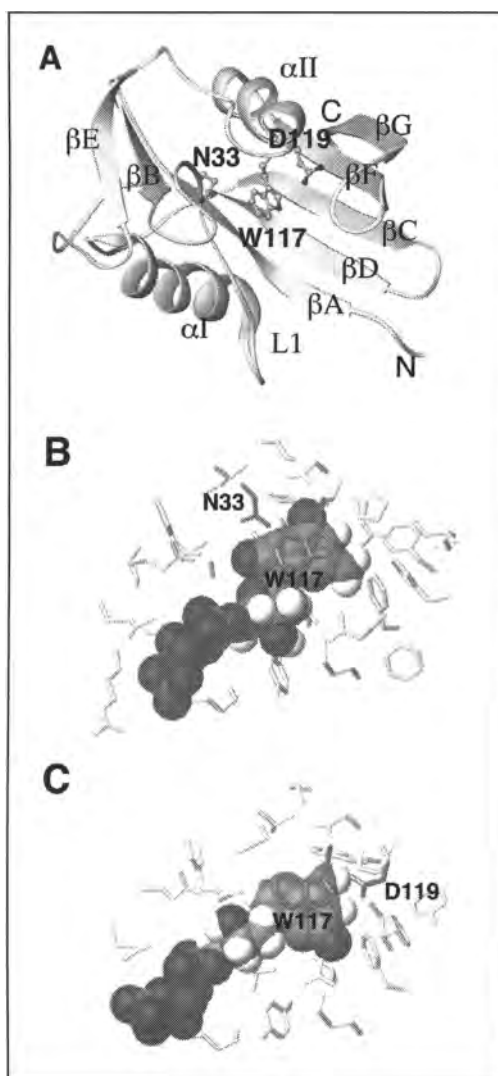


Figure 4. Structure of MTH1 and the docking model of MTH1 with 2-OH-dATP or 8-OH-dGTP. (A) Ribbon representations of MTH1 (PDB code, 1iRY) with N33, D119, W117 residues. A model of the MTH1:8-OH-dGTP (B) and the MTH 2-OH-dATP (C) complexes is shown.⁶⁹ (Courtesy of Dr. M. Mishima).

form of the purine, namely 8-OH-Gua and 2-OH-Ade, while Trp-117 is important for determining the affinity with purine rings (Fig. 4B,C).^{28,31}

Recently, by searching for proteins with the phosphohydrolase module (Fig. 3), two other mammalian proteins, MTH2 and NUDT5, were identified with the potential to hydrolyze either 8-OH-dGTP or 8-OH-dGDP to 8-OH-dGMP, respectively.^{32,33} The discovery of NUDT5 with 8-OH-dGDPase activity, further revealed that MTH1 and MutT can hydrolyze 8-OH-dGDP as well as their triphosphate forms.^{34,35} Additionally, similar to MutT, MTH1 hydrolyzes 8-OH-dGTP/8-OH-dGDP and 8-OH-GTP/8-OH-GDP to the monophosphates,

while NUDT5 only hydrolyzes 8-OH-dGDP and 8-OH-GDP. Since MTH1 also recognizes oxidized forms of dATP and ATP as mentioned above, we expect that their diphosphate forms can be hydrolyzed by MTH1, thus indicating that MTH1 is the most powerful enzyme for the sanitization of nucleotide pools (Fig. 5).

8-OH-GTP can be generated not only by the direct oxidation of GTP but also by the phosphorylation of 8-OH-GDP by nucleotide diphosphate kinase, and 8-OH-GTP thus formed can serve as a substrate for RNA polymerase II to induce transcription errors.³⁶ The degradation product of MTH1/2, NUDT5 or MutT activity, i.e., 8-OH-GMP, cannot be reutilized, since guanylate kinase, which has the potential to phosphorylate both GMP and dGMP, is inactive on 8-OH-GMP. Ribonucleotide reductase, which catalyzes the reduction of four naturally occurring ribonucleoside diphosphates, cannot convert 8-OH-GDP to 8-OH-dGDP. However, ribonucleotide reductase itself may oxidize its products, namely deoxyribonucleoside diphosphates, since it contains a stable tyrosyl radical which is a strong oxidant (Fig. 5).³⁷

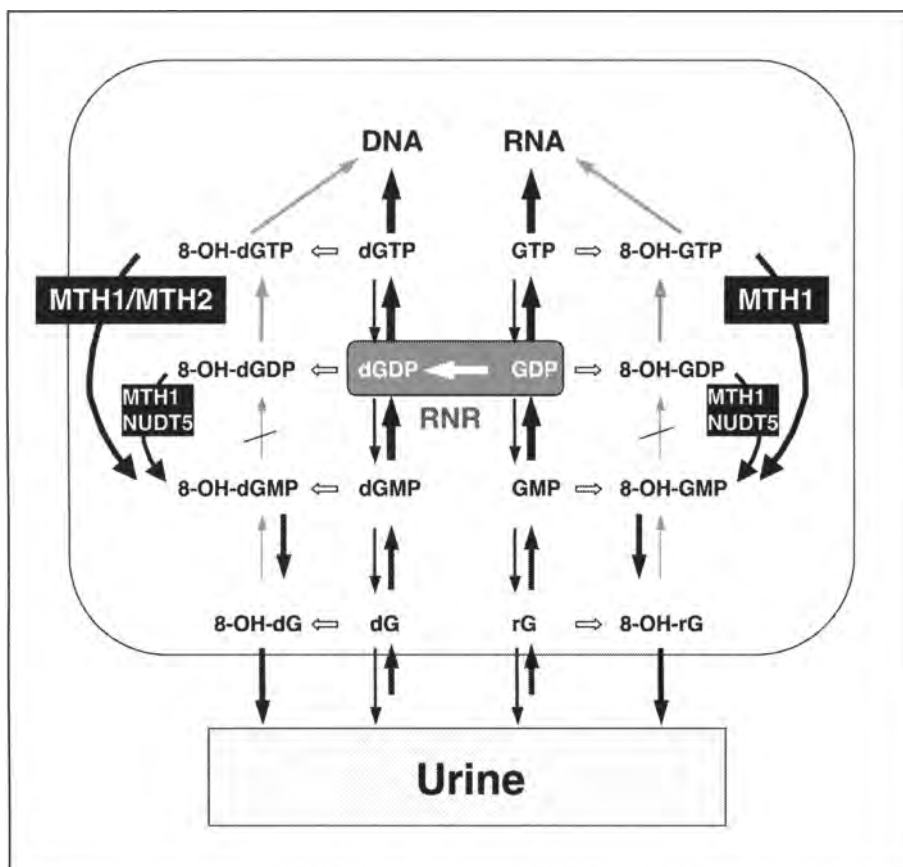


Figure 5. Sanitization of 8-OH-Gua containing nucleotides in mammalian cells. 8-OH-Gua containing nucleotides are generated by ROS, as shown by the open arrows. MTH1 protein hydrolyzes both 8-OH-dGTP and 8-OH-dGDP to 8-OH-dGMP, and also hydrolyzes 8-OH-GTP and 8-OH-GDP to 8-OH-GMP. MTH2 hydrolyzes only 8-OH-dGTP, while NUDT5 hydrolyzes 8-OH-dGDP and 8-OH-GDP to the monophosphates. 8-OH-dG 8-hydroxy-2'-deoxyguanosine; 8-OH-rG, 8-hydroxy-guanosine; RNR, ribonucleotide reductase.

Since MutT homologs are conserved from *E. coli* to humans, one can expect that all organisms should have such homologs. In *Arabidopsis thaliana*, there are more than 10 proteins with the phosphohydrolase module (AtNUDT1 to 11), and AtNUDT1 has been proven to have the ability to hydrolyze 8-OH-dGTP to 8-OH-dGMP.³⁸ We also searched for candidates with the phosphohydrolase module for the homologs of MutT/MTH1 in the yeast *Saccharomyces cerevisiae*.³⁹ Among the 5 identified candidates, a biochemical analysis indicated that YLR151c protein is capable of hydrolyzing both 8-OH-dGTP and 2-OH-dATP to the monophosphates. YLR151c was able to suppress the occurrence of A:T to C:G transversion mutations caused by the misincorporation of 8-OH-dGTP in an *E. coli mutT* deficient strain. The frequency of the spontaneous mutation in a yeast strain deficient in *YLR151c* was ~14-fold higher than that in the wild type.

Mutagenesis and Carcinogenesis Caused by the Accumulation of Oxidized Nucleotides and Prevention by MTH1

In order to elucidate the biological consequence of the cellular accumulation of oxidized nucleotides, we established MTH-null embryonic stem (ES) cells, MTH-null mice and embryonic fibroblast cell lines derived from the mice. MTH1-null ES cells exhibit a two-fold increased spontaneous mutation rate, thus confirming that MTH1 indeed plays a role in avoiding errors caused by 8-OH-dGTP or 2-OH-dATP in the nuclear genome.⁴⁰ Furthermore, MTH1-null mice exhibited a several-fold increased incidence of spontaneous carcinogenesis in the liver, with less of an increase in the lung and stomach, at about 1.5 years after birth, in comparison to wild-type mice,⁴⁰ probably because of their mutator phenotype. We also recently reported that an overexpression of human MTH1 (hMTH1) in mismatch-deficient mouse embryo fibroblasts efficiently suppresses the increased spontaneous mutagenesis, thus indicating that the oxidation of the nucleotide pool is a significant contributor to spontaneous genetic instability in mismatch repair deficient cells.⁴¹

Cellular Dysfunction Caused by the Accumulation of Oxidized Nucleotides and Its Prevention by MTH1

We recently reported that lung adenoma/carcinoma developed spontaneously in OGG1-null mice at about 1.5 years after birth, 8-OH-Gua was found to accumulate in their genomes due to the lack of excision repair of 8-OH-Gua initiated by 8-OH-Gua DNA glycosylase encoded by the *Ogg1* gene. We then found that no tumor was formed in the lungs of mice lacking both OGG1 and MTH1 proteins, despite an increased accumulation of 8-OH-Gua in these mice.⁴² This observation suggests that a *Mth1* gene disruption resulted in a suppression of the tumorigenesis caused by an OGG1-deficiency. If an accumulation of a large amount of 8-OH-dGTP and 8-OH-GTP in nucleotide pools, or 8-OH-Gua in cellular DNA and RNA, results in cell death, one can expect that cancer stem cells lacking both OGG1 and MTH1 proteins may not survive to produce progenitors with mutations in either proto-oncogenes or tumor suppressor genes, thus suppressing carcinogenesis in mice.

Indeed, MTH1-null mouse embryo fibroblasts (MEF) were shown to be highly susceptible to cell dysfunction and death caused by exposure to H₂O₂, with morphological features of pyknosis and electron dense deposits accumulated in the mitochondria. The cell death observed was not dependent on either poly (ADP-ribose) polymerase or caspases. A continuous accumulation of 8-OH-Gua, both in the nuclear and mitochondrial DNA, was observed after exposure to H₂O₂. All of the H₂O₂-induced alterations observed in MTH1-null MEFs were effectively suppressed by the expression of wild-type human MTH1 (hMTH1) (Fig. 6), while they were only partially suppressed by the expression of mutant hMTH1 which possessed either 8-OH-dGTPase or 2-OH-dATPase activity.⁴³ MTH1 thus protects the cells from H₂O₂-induced cell dysfunction and death by hydrolyzing oxidized purine nucleotides. These results explain why mice lacking both OGG1 and MTH1 developed dramatically fewer lung tumors than OGG1-null mice.

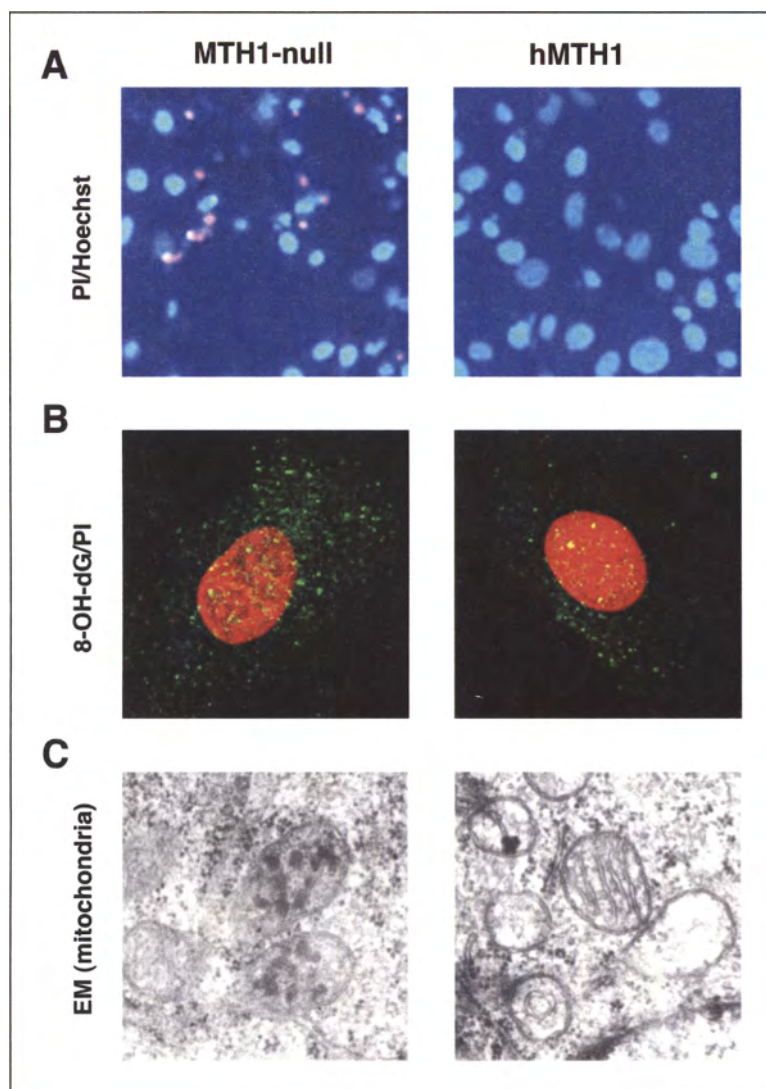


Figure 6. MTH1 protects the cells from oxidative stress. A) The cells exposed to 500 μ M H_2O_2 for 24 h were stained with Hoechst33342 and propidium iodide (PI). B) Immunofluorescence microscopy with anti-8-hydroxy-2'-deoxyguanosine (8-OH-dG) antibody 8hr after exposure to H_2O_2 . C) Cells exposed to H_2O_2 for 24 h were examined by electron microscopy. All of the H_2O_2 -induced alterations observed in MTH1-null MEFs (MTH1-null) were effectively suppressed by the expression of wild-type human MTH1 (hMTH1).

The increased expression of MTH1 protein in human tumor tissues, including brain tumors, kidney, lung and liver cancers, is accompanied by an increased accumulation of 8-OH-Gua in the malignant tissues or surrounding normal tissues,⁴⁴⁻⁴⁷ thus indicating an increased degree of oxidative stress in the tumor tissues. In glioma cases, the levels of MTH1 expression and 8-OH-Gua accumulation correlated significantly with the grade of malignancy in glioma, suggesting that MTH1 may protect cancer cells against increased oxidative stress.⁴⁵ Recently, Spenia

et al⁴⁸ reported that among the several different components contributing to the maintenance of 8-OH-Gua levels in human DNA, the greatest contributor is the removal of 8-OH-dGTP from the cellular nucleotide pool by hMTH1, especially in non small cell lung cancer patients. We may thus conclude that cancer cells exposed to higher oxidative stress require an increased expression of MTH1 for their survival. It is noteworthy that OGG1 is easily inactivated under oxidative conditions,⁴⁹ however, we did not observe an increased expression of OGG1 in cancer tissue specimens, possibly for this very reason.

Since MTH1 efficiently protects cells from oxidative stress, we propose that the compounds which suppress the MTH1 function or expression, must thus be new candidates for cancer chemotherapy, to sensitize cancer cells against certain types of anticancer drugs. The structure of the substrate-binding pocket of hMTH1 and its catalytic mechanism, together with MTH1-null mouse embryo fibroblasts expressing hMTH1, provide the theoretical basis for both the drug design and the development of practical screening systems for such compounds.

Neuronal Accumulation of Oxidized Nucleotides Causes Neurodegeneration, Suppression by MTH1

Oxidative DNA damage, such as 8-OH-Gua, has been demonstrated to accumulate in both nuclear and mitochondrial genomes during aging, and such accumulation appears to increase dramatically in patients with various neurodegenerative diseases, such as Parkinson's disease (PD),^{50,51} Alzheimer's disease (AD)⁵² or amyotrophic lateral sclerosis (ALS).⁵³

We have shown that a significant increase of 8-OH-Gua in the cytoplasm or mitochondria was accompanied with a coincidentally elevated expression of MTH1 and the mitochondrial form of OGG1 (OGG1-2a)⁵⁴ in the substantia nigral neurons of PD patients.^{50,55} In post-mortem tissue specimens from patients with AD, the expression levels of MTH1 in the entorhinal cortex were also elevated, whilst the levels of MTH1 apparently decreased in the stratum lucidum at CA3, corresponding to mossy fiber synapses, where MTH1 is highly expressed in the control subjects.⁵⁶ In contrast, OGG1-2a was found to decrease in the orbitofrontal gyrus and the entorhinal cortex in AD patients in comparison to that in control cases.⁵⁷ The accumulation of 8-OH-Gua was increased in a majority of the large motor neurons in ALS cases with a decreased expression of OGG1-2a but not MTH1.⁵³ It is thus likely that OGG1-2a is indeed unstable under increased oxidative stress, in comparison to MTH1.

We recently found the levels of 8-OH-Gua in cellular DNA and RNA to increase in the mouse nigrostriatal system during tyrosine hydroxylase (TH)-positive dopamine neuron loss induced by the administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). In contrast to wild type mice, MTH1-null mice exhibited a greater accumulation of 8-OH-Gua in mitochondrial DNA, accompanied by a more significant decrease in TH- and dopamine transporter-positive fibers in the striatum after MPTP administration (Fig. 7).⁵⁸ We thus demonstrated that MTH1 indeed protects the dopaminergic neurons from oxidative damage to nucleic acids, especially the mitochondrial DNA of striatal nerve terminals of dopaminergic neurons.

Oxidative Deamination of Nucleotides and Its Biological Consequences

The two purine bases present in DNA molecules have an amino group in their structure. Oxidative deamination converts adenine and guanine to hypoxanthine and xanthine, respectively. For example, exposure of human cells to nitrite leads to the intracellular generation of reactive nitrogen species, inducing the deamination of purines in cellular DNA.⁵⁹ Deoxyinosine triphosphate (dITP), a hypoxanthine-containing nucleotide, can be inserted opposite cytosine in the DNA template, and any dNTP can be incorporated opposite hypoxanthine in template DNA by DNA polymerase I,⁶⁰ thus inducing mutations. Similar to 8-OH-Gua, it is considered that hypoxanthine in genomic DNA arises from two independent pathways: one is a consequence of the direct deamination of adenine bases in DNA, while the other is a consequence of the incorporation of dITP, which is generated in the resident nucleotide pool, during

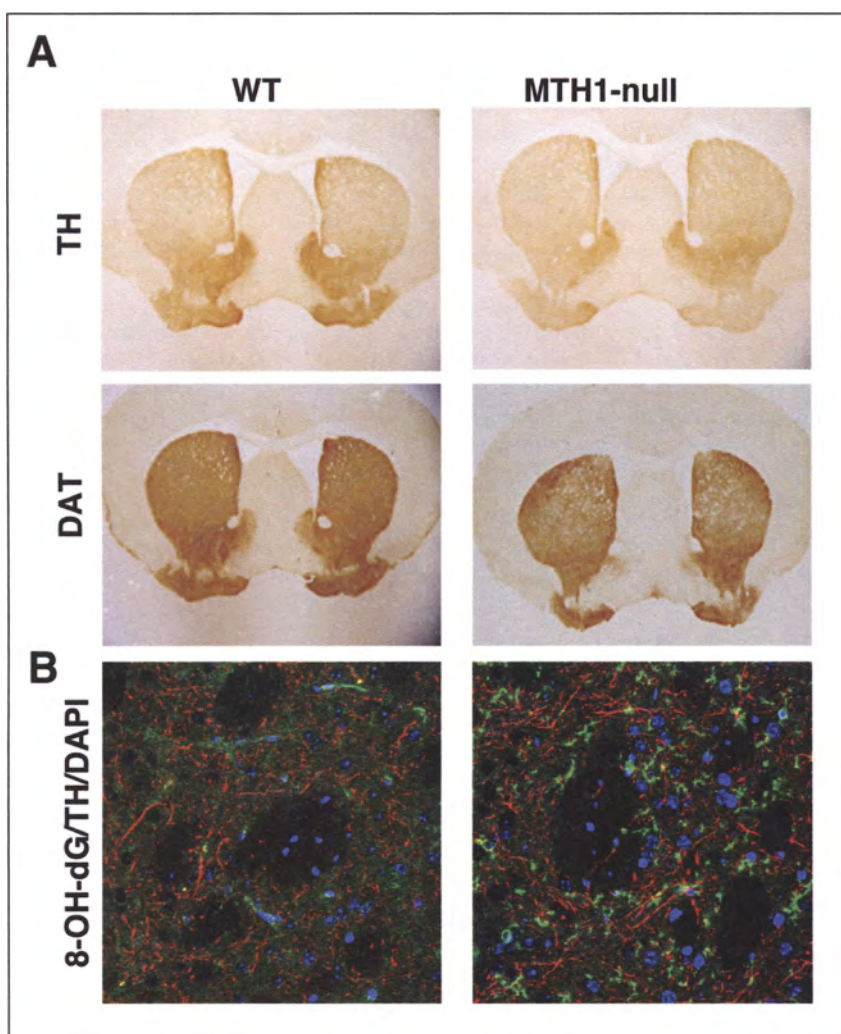


Figure 7. MTH1 protects dopaminergic neurons from MPTP-induced oxidative stress. A) Striatal immunoreactivities for TH and DAT were examined in the sections prepared from the mice administered MPTP. MPTP (30 mg/kg) was administered i.p. to wild-type and MTH1-null mice once a day for 5 consecutive days. At 7 days after the last injection, the mice were sacrificed for the analyses. B) The localization of 8-hydroxy-2'-deoxyguanosine (8-OH-dG) in the striatal terminal fibers of dopamine neurons determined by laser scanning confocal microscopy. The sections prepared from wild-type and MTH1-null mice 12 h after MPTP injection (30mg/kg, i.p.) were stained for 8-OH-dG in DNA with the N45.1 mAb (green), nuclear DNA with DAPI (blue), TH (red), and their merged images are shown. The sections were pretreated only with RNase.

DNA synthesis. To prevent the latter pathway, organisms possess inosine triphosphate pyrophosphatase (ITPase), which hydrolyzes deaminated purine nucleoside triphosphates, such as ITP, dITP and XTP^{61,62}

In *E. coli*, ITPase is encoded by the *rdgB* gene. In contrast to MutT or MTH1, ITPase lacks the phosphohydrolase module, and it acts as a homodimer. A mutant lacking the *rdgB* gene is

viable but it shows synthetic lethality with *recA* or *recBC* mutation.⁶³ The lethality of *rdgB recA* or *rdgB recBC* double mutants can be suppressed by the inactivation of endonuclease V which initiates the excision of deoxyinosine (dI) or deoxyxanthosine (dX) incorporated into DNA.⁶⁴ It is likely that an ITPase deficiency results in the accumulation of its substrate nucleotides, dITP or dXTP in the nucleotide pool, thus causing an increased accumulation of dI or dX in DNA, and a further excision repair initiated by endonuclease V leads to chromosomal fragmentation in *recA* or *recBC* mutants. A deficiency in ITPase in a human was first reported to have an elevated ITP level in erythrocytes as an inherited abnormality.⁶⁵ However, such individuals do not exhibit any abnormal phenotype even with an accumulation of ITP in the erythrocytes. A cDNA for human ITPase was isolated and its gene, *ITPA*, was identified,⁶¹ and the structure of the *hITPA* gene and nucleotide alterations responsible for the ITPase deficiency were reported.⁶⁶ In the adult mouse, the *ITPA* gene is strongly expressed in the brain and testes, although mRNA was observed in all types of tissue. Mouse ITPA protein is expressed in postmitotic neurons as well as in proliferative tissues.⁶⁷ It is known that nitric oxide, a neurotransmitter, promotes the deamination of ATP/dATP or GTP/dGTP,⁶⁸ thus increasing the intracellular concentration of ITP/dITP or XTP/dXTP in the brain. The function of ITPase may thus be important to eliminate the deamination products of dATP/ATP and dGTP/GTP in the brain, as they can be substrates for DNA and RNA synthesis, whilst also acting as cofactors in many kinds of protein function.

Conclusion

The availability of MTH1-null cells or mice together with the quantitative and in situ detection of 8-OH-Gua has significantly improved our understanding of the biological consequences of their accumulation. However, other oxidized nucleotides such as 2-OH-dATP/2-OH-ATP whose quantitative measurements have not yet been established, may have even more of an impact on human diseases. The discovery of an enzyme that specifically hydrolyzes dITP/ITP or dXTP/XTP, and which are products of the oxidative deamination of dATP/ATP or dGTP/GTP, further emphasizes the impact of the oxidation of nucleotides on cellular dysfunction. In this chapter, we did not discuss oxidized pyrimidine nucleoside triphosphates, since there has yet to be a report describing an enzyme(s) which hydrolyzes them.

Acknowledgements

We extend our special thanks to all other members in our laboratory and to Drs. M. Takahashi, N. Hattori, T. Iwaki, M. Shirakawa, M. Mishima, H. Kamiya, H. Kasai, M. Furuichi, H. Hayakawa, Y. Nakatsu, T. Tsuzuki, H. Maki and M. Sekiguchi for helpful discussions, and to Dr. B. Quinn for useful comments on this manuscript.

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Nucleotide Incision Repair: An Alternative and Ubiquitous Pathway to Handle Oxidative DNA Damage

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Abstract

Aerobic respiration and exogenous factors such as ionizing radiation and drugs generate reactive oxygen species (ROS). DNA has limited chemical stability and it is one of the most biologically important targets of ROS.¹ Oxidative DNA lesions are believed to be a major type of endogenous damage leading to human degenerative disorders including cancer, cardiovascular disease and brain dysfunction. The clinical features of inherited human DNA repair deficiency disorders such as Cockayne syndrome and Fanconi's anemia point to the complex nature of endogenous oxidative DNA damage which may include bulky adducts, interstrand crosslinks and clustered lesions. Oxidized DNA bases are substrates for two overlapping pathways: base excision repair (BER) and nucleotide incision repair (NIR). In the BER pathway, a DNA glycosylase cleaves the *N*-glycosylic bond between the modified base and deoxyribose, leaving either an abasic site or a single-strand break in DNA.² Alternatively, in the NIR pathway, an apurinic/apyrimidinic (AP) endonuclease incises oxidatively damaged DNA in a DNA glycosylase-independent manner, providing the correct ends for DNA synthesis, coupled to the repair of the remaining 5'-dangling modified nucleotide.³ We have demonstrated that the major human apurinic/apyrimidinic (AP) endonuclease (Ape1) is involved in the NIR pathway.⁴ NIR and BER pathways share many common substrates suggesting that they work in concert to cleanse genomic DNA of potentially mutagenic and cytotoxic lesions. Recently, we have genetically separated AP endonuclease and nucleotide incision activities to demonstrate that NIR handles a distinct type of oxidative DNA damage that cannot be processed in the BER pathway.⁵ The aim of this review is to summarise the present knowledge about the alternative DNA repair pathways for oxidised base modifications.

Introduction

ROS generate nonbulky DNA base lesions, consequently, base excision repair (BER), initiated by DNA glycosylases is thought to be the major pathway for the removal most of these oxidized bases (Fig. 1).² Despite the progress in understanding the molecular mechanisms of base damage recognition and excision it is still unclear why mice, deficient in DNA glycosylases that remove oxidized bases, are not sensitive to oxidizing agents.⁶ The BER pathway,⁷ which requires the sequential action of two enzymes for proper incision of DNA,⁸

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raises theoretical problems concerning the efficient repair of oxidative DNA damage, because genotoxic intermediates are generated. These include apurinic/apyrimidinic (AP) sites, 2'-deoxyribose-5'-phosphate and/or blocking 3'-termini groups that must be eliminated by additional steps before initiating DNA repair synthesis. Genetic data indicate that mutants lacking oxidized base-specific DNA glycosylases/AP lyases are not sensitive to oxidizing agents and ionizing radiation.⁹⁻¹² Although, some exceptions to this phenomenon may exist.¹³⁻¹⁵ Recent studies have indicated that single and double DNA glycosylase deficient mouse strains are not more sensitive to oxidising agents.^{6,16,17} This is in striking contrast to the highly sensitive phenotype of AP endonuclease deficient bacterial, yeast and mammalian mutant cells towards oxidizing agents and ionizing radiation.¹⁸⁻²¹ Therefore, this biological evidence hints at the existence of an alternative, back-up, repair pathway. In the mid- 1990s, it was shown that certain types of DNA damage, such as UV-induced DNA photoadducts and alpha-anomeric nucleotides, can be repaired in a DNA glycosylase-independent manner by damage-specific endonucleases.^{22,23} Recent findings that *Escherichia coli* endonuclease IV (Nfo) and Nfo-like endonucleases nick DNA on the 5' side of various oxidatively damaged bases, generating 3'-hydroxyl and 5'-phosphate termini, indicate the existence of an alternative pathway to the classic BER pathway.³ It has been proposed to name it the nucleotide incision repair (NIR) pathway (Fig. 1). The proposed NIR pathway which can eliminate the genotoxic intermediates, explains the genetic data and most likely identifies the physiological and original target as the modified base and not an artificial reduced abasic site, for the long patch repair pathway described in human cells.²⁴ This alternative repair pathway is evolutionarily conserved from *E. coli* to humans.⁴

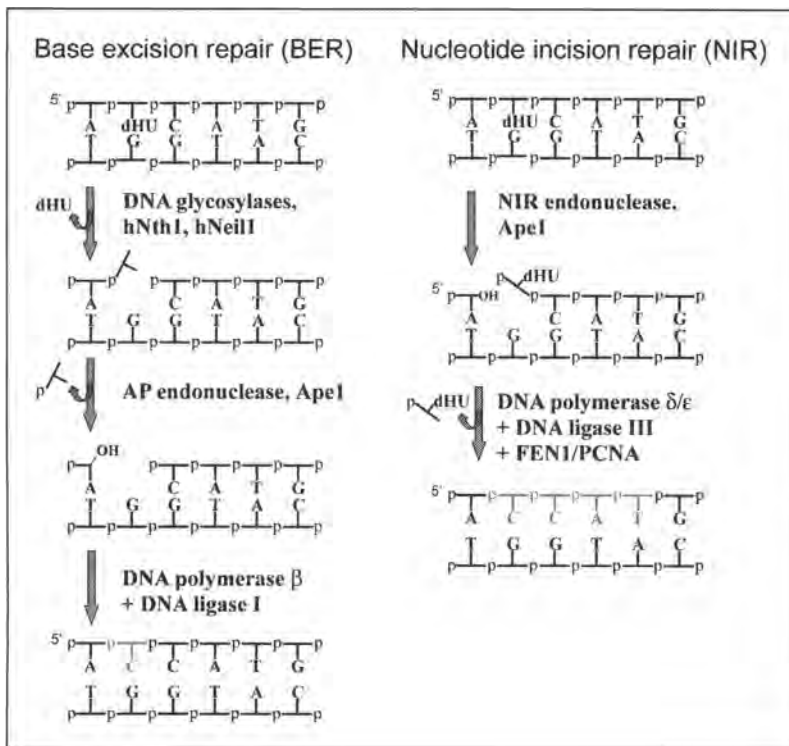


Figure 1. General models of the base excision and nucleotide incision repair pathways for oxidative DNA damage.

Free Radical Species and Oxidative Damage of DNA

ROS can damage nucleobases and sugar units in DNA either directly, or indirectly. At present, more than 100 different base and sugar modifications induced by free radicals have been identified.²⁵ Hydroxyl radicals, which are the most active species, predominantly, react with the C₅-C₆ double bond of pyrimidines forming thymine and uracil glycols (Tg and Ug), 5-hydroxyuracil (5ohU) and 5-hydroxycytosine (5ohC)²⁶ and with the C₈ of purines forming 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxoG)²⁷ (Fig. 2, not shown, E, H and G). Indirectly, ROS generate reactive aldehydes, a product of membrane lipid peroxidation, which in turn react with DNA bases forming exocyclic adducts.²⁸ The alpha-anomer of 2'-deoxyadenosine (α dA) and 5,6-dihydrouridine (DHU) residues constitute the major DNA adducts generated by ionizing radiation (IR) under anoxic conditions²⁹⁻³¹ (Fig. 2B,C). DHU is a reduction product, which is generated by the reaction of C₅-C₆ with either hydrogen atoms or hydrated electrons and protons. The alpha-anomers of 2'-deoxynucleosides (α dN) including α dA, α T and α dC are generated by abstraction of the anomeric hydrogen atom at C1' by hydroxyl radicals.³² These radicals lead to epimerization at the C1' atom either by disproportionation or by addition of a hydrated electron and subsequent protonation.³³ The α dN residue constitutes a moderate replication block both in vitro and in vivo, and generates exclusively a single nucleotide deletion in vivo.³⁴ C $\rightarrow$ T transitions and G $\rightarrow$ T transversions are the most common point mutations occurring in tumour suppressor genes commonly mutated in human cancers.³⁵ These types of substitution could arise from mispairing of either 8-oxoG or cytosine-derived lesions such as DHU, Ug, 5ohU and 5ohC with adenine.^{36,37} Interestingly, the steady state levels of Ug and 5ohC residues in DNA from mammalian tissues and human cells are higher than those observed with 8-oxoG.³⁸

Environmental exposure to IR and drugs may lead to oxidative base and sugar modification, as well as strand breaks in the genome. Severe biological effects of IR and drugs such as bleomycin, mitomycin and neocarzinostatin are correlated with the complexity of induced DNA damage. In fact, base and/or sugar modifications, as well as strand breaks, could be closely spaced forming so called clusters, i.e., more than one lesion located within one helical turn on the same strand or on the opposite strand.³⁹ Importantly, oxidized base clusters can be induced by normal oxidative metabolism in the cells.⁴⁰ It is anticipated that the excision of oxidatively damaged bases, including AP sites, on both strands will, if not tightly regulated, either inhibit certain steps of repair or produce double strand breaks and thus be lethal for the cells.⁴¹⁻⁴³ Clusters are postulated to be critical because they may be more difficult to repair than single lesions. Therefore, oxidative DNA damage clustering might be a key factor in the biological consequences of oxidative stress. However, at present, the repair mechanisms for the clusters are poorly understood.

AP Endonucleases Involved in the Nucleotide Incision Repair Pathway

Escherichia coli has two AP endonucleases: exonuclease III (Xth) and endonuclease IV (Nfo). Nfo is an EDTA-resistant multifunctional enzyme with AP endonuclease, 3'-phosphatase and 3'-phosphoglycoaldehyde diesterase activities.⁴⁴ The 30 kDa protein contains three Zn²⁺ ions in the active site which are critical for its function.^{45,46} The *nfo* gene is under the control of the *saxRS* system and inducible by O₂⁻ and *NO radicals.⁴⁷ *E. coli nfo* mutants are hypersensitive to an anticancer drug, bleomycin, as well as to the organic peroxide, *tert*-butyl-hydroperoxide (*t*-BuO₂H), under conditions of chronic exposure to these drugs¹⁸ and to macrophage generated *NO radicals.⁴⁸ In addition, the double *nfo xth* mutants are more sensitive to γ -radiation, H₂O₂ and alkylating agents compared to *nfo* and *xth* single mutants. Thus, Nfo operates in both pathways: it repairs specific classes of oxidative damage and serves as a backup activity for Xth in repairing AP sites and 3'-blocking termini. Importantly, Nfo, but not Xth, is involved in the NIR pathway and able to process a large variety of DNA lesions generated by oxidative stress such as DHU, 5,6-dihydrothymidine (DHT), 5ohU, 2,6-diamino-4-hydroxy-5-N-methylformamidopyrimidine (me-FapyGua), α dA and α T (Fig. 2).^{3,22,49}

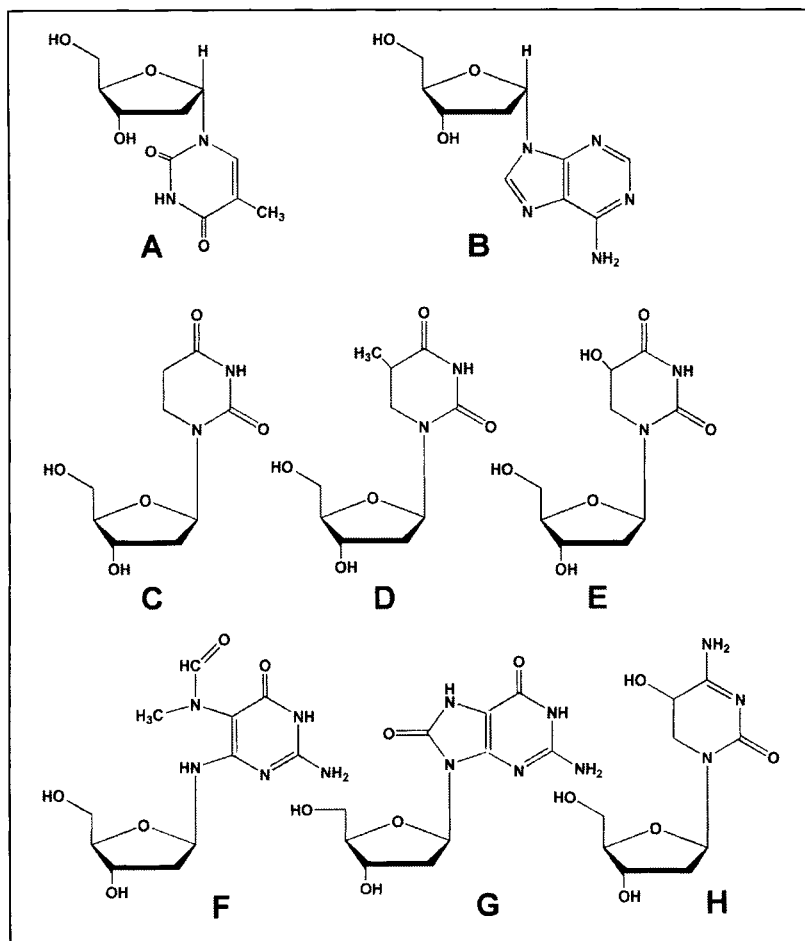


Figure 2. Chemical structures of some major oxidative DNA base damage. A) alpha-anomer of thymidine (α T); B) alpha-anomer of 2'-deoxyadenosine (α dA); C) 6-dihydrouracil (DHU); D) 5,6-dihydrothymidine (DHT); E) 5-hydroxyuridine (5ohU); F) 2,6-diamino-4-hydroxy-5-N-methylformamidopyrimidine (me-FapyGua); G) 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxoG); H) 5-hydroxyuridine (5ohC).

Saccharomyces cerevisiae AP endonuclease, Apn1, is a counterpart of *E. coli* Nfo, and together with other members constitutes the Nfo family of DNA repair enzymes. Apn1 is a multifunctional 41.4 kDa protein localized in the nucleus, as well as in the mitochondria.^{50,51} Apn1 accounts for > 90% of the total AP endonuclease activity in yeast.⁵² Like Nfo, Apn1 is a metalloenzyme that has a 3'→5' exonuclease activity and NIR and 3'-diesterase activity, which removes a multitude of 3'-blocking groups at the single-strand breaks in DNA (e.g., 3'-phosphoglycolate and 3'-phosphate) which are induced by oxidative agents.^{53,54} The yeast mutants lacking Apn1 (*apn1Δ*) are hypersensitive to both oxidative (H_2O_2 and *t*-BuO₂H) and alkylating (methyl- and ethylmethane sulfonate) agents and have a 6- to 12-fold higher rate of spontaneous mutations than wild-type.²¹ The *apn1Δ* mutants exhibit a mutator phenotype characterized mainly by a 60-fold increase in the A•T to G•C transversion rate.⁵⁵ *S. cerevisiae* also has a second AP endonuclease (Apn2) belonging to the Exo III family, and loss of this enzyme dramatically increases the sensitivity to genotoxic agents and the spontaneous

mutation rate of *apn1Δ* cells.²⁰ Apn2 contains 3'-diesterase and 3'→5' exonuclease activities, which are 30- to 40-fold higher than its AP endonuclease activity.⁵⁶

The NIR pathway is evolutionarily conserved from *E. coli* to human cells where the major AP endonuclease, Ape1/Ref-1/HAP-1 also incises duplex oligonucleotides containing DHU and αA residues.⁴ Previously, Ape1 was independently discovered as an AP endonuclease homologous to the *E. coli* Xth protein⁴⁷ and as a redox-regulator of the DNA binding domain of Fos-Jun, Jun-Jun, AP-1 and p53 proteins as well as several other transcription factors.⁵⁷ Ape1 plays a central role in both short-patch and long-patch BER.⁵⁸ It also exhibits other DNA repair activities: 3'→5' exonuclease, phosphodiesterase, 3'-phosphatase and RNase H.⁴⁷ The X-ray structure of full-length Ape1, determined from a crystal grown at pH 7.5, reveals two metal ions bound 5 Å apart in the active site.⁵⁹ It has been proposed that the binding of a divalent cation to carboxylates of residues Asp70 and Glu96 is required for catalysis, while the binding of a second divalent cation to residues Asp210, Asn212 and His309 renders the enzyme efficient for its AP-endonuclease function by increasing the velocity of the reaction product release.^{60,61} Interestingly, the structure of free Ape1 at acidic pH has only one bound metal ion.⁵⁹ We have demonstrated that Mg²⁺ cations and pH induce conformational changes and modulate the substrate specificity of Ape1 in an apparently allosteric manner.⁴ At low concentration of divalent cations and pH, Ape1 binds strongly to both DNA substrate and reaction product and becomes active towards NIR substrates. These observations may suggest the existence of at least two conformations of Ape1: NIR-proficient and -inactive forms.

A second AP endonuclease, Ape2, homologous to the *S. cerevisiae* Apn2, was identified in mammals.⁶² The purified recombinant Ape2 protein exhibits strong 3'→5' exonuclease and 3'-phosphodiesterase activities and has only a very weak AP-endonuclease activity.⁶³ Interestingly, the Ape2-catalyzed 3'-5' exonuclease preferentially acts at mismatched deoxyribonucleotides at the recessed 3'-termini of a partial DNA duplex. APEX2-null mice exhibit growth retardation and a relatively severe defect in lymphopoiesis accompanied by a G2/M phase arrest.⁶⁴ Based on these observations Nakabeppu and colleagues hypothesized that the human diseases anticipated from an hAPE2 deficiency might be associated with decreased body size, moderate immunodeficiency, and a high sensitivity of lymphocytes to X-ray irradiation.⁶⁴

Substrate Specificity of NIR Endonucleases and Their Physiological Relevance

E. coli, Nfo, *S. cerevisiae* Apn1 and human Ape1 can initiate the NIR pathway by nicking DNA on the 5' side of alpha-anomeric deoxynucleotides (αT, αA and αC) (Fig. 2A,B and not shown), DHU (Fig. 2C), DHT (Fig. 2D), 5ohU (Fig. 2E), me-FapyGua (Fig. 2F) and 5ohC (Fig. 2H) residues. Except for αdN residues, all these damaged bases are also substrates for the DNA glycosylases. Measurements of the apparent k_{cat}/K_M values for AP endonucleases on oxidatively damaged nucleotides indicate that the preferred substrate for all these enzymes was the AP site (THF residue) (Table 1). But the catalytic efficiency (k_{cat}/K_M) of Apn1 (from 0.07 to 0.76 min⁻¹ nM⁻¹) and Nfo (from 0.58 to 2.1 min⁻¹ nM⁻¹) on αdN are of the same order as on THF (0.48 - 2.0 min⁻¹ nM⁻¹ for Apn1 and 0.58 - 1.2 min⁻¹ nM⁻¹ for Nfo). Comparison of the kinetic parameters for purified enzymes suggests that Nth and hNth1 cleaves the duplex oligonucleotides containing a single oxidative DNA base damage with similar efficiency to that of Nfo, Apn1 and Ape1 (Table 1). However, comparison of the BER and NIR activities, in *E. coli*, *S. cerevisiae* and human cell-free extracts, suggests that the majority of oxidative DNA base damage can be processed in a DNA glycosylase-independent manner.^{4,65}

Previously, it was shown that a single amino acid change can modify the AP endonuclease substrate specificity arguing that subtle alterations in the protein conformation may change its catalytic properties.^{66,67} The truncated Ape1 (NΔ61-Ape1) lacking the first 61 N-terminal amino acids has reduced NIR but normal AP endonuclease activity suggesting that DNA repair functions of the AP endonuclease can be genetically separated.⁴ The crystal structure of Nfo complexed to duplex DNA containing an AP site shows that three Zn²⁺ ions are bound in

Table 1. Kinetic constants for the incision of duplex oligonucleotides containing various lesions paired with different bases by the bacterial, yeast and human AP endonucleases^a

Enzyme	Substrate	K_M , nM ^a	k_{cat} , min ⁻¹	k_{cat}/K_M , min ⁻¹ nM ⁻¹	References
Nfo	3'-PGA ^b	7.5 ± 1.0	57 ± 1.8	7.5	Ishchenko et al ⁴⁹
	3'-p ^b	6.7 ± 1.4	47 ± 2.5	6.9	
	αT•A	7.5 ± 3.0	11.0 ± 0.2	1.5	
	THF•A	3.8 ± 0.8	4.5 ± 0.2	1.2	
	THF•T	16.2 ± 2.0	9.3	0.58	Ishchenko et al ⁵
	αdA•T	24 ± 2.1	14 ± 0.3	0.58	
	DHT•A	13 ± 2.0	2.0 ± 0.1	0.15	
	DHU•G	41 ± 4.1	4.6 ± 0.2	0.11	
	5ohU•G	71 ± 11.0	0.9 ± 0.06	0.013	Ishchenko et al ⁴⁹
	me-FapyG•C	290 ± 50	2.2 ± 0.1	0.008	
Apn1	THF•C	13 ± 1.2	26 ± 2.0	2.0	Ishchenko et al ⁴⁹
	THF•G	24 ± 4.4	11 ± 1.0	0.48	
	αT•A	6.5 ± 1.7	3.1 ± 0.2	0.48	
	αT•C	14 ± 2.0	3.5 ± 0.08	0.25	
	αdA•T	17 ± 2.1	1.2 ± 0.1	0.07	Ishchenko et al ⁶⁵
	me-FapyG•C	50 ± 10	0.7 ± 0.1	0.014	
	5ohU•G	26 ± 3	0.34 ± 0.01	0.013	
	DHU•G	103 ± 10	0.96 ± 0.03	0.01	
Ape1	DHT•A	23 ± 3	0.2 ± 0.01	0.009	Gros et al ⁴
	THF•G ^c	19 ± 2.0	30	1.6	
	THF•G ^d	2.7 ± 0.4	0.12	0.045	
	DHU•G ^d	8.4 ± 1.8	0.16	0.02	
	αA•T ^d	7.2 ± 1.6	0.12	0.016	
Nth	DHU•G	15 ± 3	0.34 ± 0.02	0.023	Ishchenko and Saporbaev ³
hNth1	DHU•G	47	0.6 ± 0.08	0.013	

a) For K_M and k_{cat} determination, the linear velocity was measured and the constants were determined from Lineweaver-Burk plots. b) DNA strand breaks containing 3'-phosphoglycolate (3'-PGA) and 3'-phosphate ends. All other lesions are within full duplex DNA and not associated with strand break. c,d) Enzyme activity was measured under standard reaction conditions for AP endonuclease^b or NIR^c assay.

the protein by conserved residues that cluster at the center of a crescent-shaped cavity.⁴⁶ Site-directed mutagenesis of the conserved amino acid residues support participation of Zn^{2+} ions in catalysis of phosphodiester bond cleavage.⁶⁸ Recently, we succeeded in the mutational separation of BER and NIR functions of Nfo by constructing three mutants carrying missense mutations histidine 69 → alanine (Nfo-H69A), histidine 109 → alanine (Nfo-H109A) and glycine 149 → aspartic acid (Nfo-G149D).⁵ All mutants were proficient in the AP endonuclease and 3'-repair diesterase activities but deficient in NIR. Analysis of the metal content reveals that all three mutant proteins have lost one of their intrinsic zinc atoms.⁵ Expression of the *nfo* mutants in a repair-deficient strain of *E. coli* complemented its hypersensitivity to alkylation (methylmethanesulfonate treatment) but not to oxidative DNA damage (*t*-BuO₂H and bleomycin treatments).⁵ The differential drug sensitivity of the mutants suggests that the NIR function handles lethal oxidative DNA damage which cannot be removed in the BER pathway.

The physiological significance of the DNA repair function is supported by the findings that Ape1 null cells display increased sensitivity to γ -irradiation. Also, strong downregulation of Ape1 stopped cell proliferation and activated apoptosis, indicating a deficiency in the repair of exogenous and endogenous DNA lesions.^{69,70} Based upon the lack of a readily discernable phenotype of DNA glycosylase-deficient mice and the increased susceptibility to oxidative stress of Ape1 heterozygous null mutant mice,⁷¹ we proposed that the novel NIR function of Ape1 serves as a back-up pathway for BER. The inhibition of Ape1 NIR activity by high Mg^{2+} concentration in vitro raises the question of whether the intracellular environment would be compatible with this function. The physiological relevance of Ape1-NIR activity in human cells was examined using the fluorescence quenching mechanism of molecular beacons.⁷² In the molecular beacon containing a single αA residue, the fluorophore is held in close proximity to the quencher by the stem-loop structure design of the oligonucleotide. We have shown that in HeLa cells, Ape1-catalyzed incision of the beacon at αA , lead to separation of the fluorophore from the quencher resulting in an increase in fluorescence.⁵ Thus, this observation strongly argues for the compatibility of the intranuclear environment of human cells with the Ape1-catalyzed NIR activity.

Structure and 3'-5' Exonuclease Activity of AP Endonucleases

Co-crystal structures of Nfo bound to the cleaved AP site analog, tetrahydrofuran, showed that the enzyme kinks the DNA helix $\sim 90^\circ$ and flips the target AP site and its opposing nucleotide out of the DNA base stack.⁴⁶ Interestingly, the Nfo active site pocket sterically excludes binding of normal β -configuration nucleotides, but it can fit α -anomeric nucleotides. In contrast, co-crystal structures of Ape1 bound to abasic DNA show that the enzyme kinks the DNA helix and binds a flipped-out AP site in a pocket that excludes DNA bases.⁷⁴ However, these and other DNA glycosylase based-models for DNA damage recognition are unable to explain the ability of Nfo and also yeast and human AP endonucleases to accommodate modified bases in the β -configuration such as DHU^{3,4} and 3,*N*⁴-benzetheno-dC⁷⁵ in the same active site. It is evident that the mechanism of action of a DNA glycosylase is fundamentally different from that of Nfo-like AP endonucleases. To access the C1' atom, DNA glycosylases flip-out the abnormal base into a specific pocket, instead the AP endonucleases target the phosphodiester bond in DNA and may access it by drastic kinking of the helix and flipping-out the opposite base. Therefore, we suggest that reduced NIR activity of the Nfo and Ape1 mutant proteins might correlate with reduced binding and insufficient bending of the duplex DNA. It is tempting to speculate that the previously described structure of native Nfo bound to a cleaved AP site with the double-nucleotide flipping and 90° DNA helix bend actually may closely resemble the NIR complex of Nfo and Ape1 bound to a damaged base. Further structural studies are required to fully investigate these phenomena.

The Nfo, Ape1 and Ape1 proteins contain progressive 3'→5' exonuclease activity which is as efficient as their abasic site cleavage activity. The fact that the exonuclease function is evolutionarily conserved among the major AP endonucleases of various origins underscores the importance of this activity in DNA repair and genome stability. Indeed, Cheng et al⁷⁶ have shown that Ape1 removes the 3'-terminal nucleotide from mismatched pairs much more efficiently than from matched pairs. The authors proposed that Ape1 may ensure fidelity of the BER pathway in vivo, since DNA polymerase β lacks a proofreading exonuclease activity.⁷⁷ Furthermore, we have demonstrated that the 3'→5' exonuclease of *S. cerevisiae* Ape1, a functional yeast counterpart of Ape1, removes normal and oxidized nucleotides 3' to a gap and nick in duplex DNA, thus providing an alternative pathway to repair 8-oxoG residues in yeast.⁷⁸ Several factors may lead to the formation of DNA lesions containing 8-oxoG residues next to strand breaks. In the BER pathway, DNA polymerase β lacking proofreading activity may generate a 3'-terminal 8-oxoG by misincorporation of 8-oxodGMP.^{79,80} Inefficient ligation of the phosphodiester bond next to 3'-terminal 8-oxoG will result in persistence of single-strand breaks with 8-oxoG residues. Similar clustered lesions may also be generated by

ionizing radiation.⁸¹ As a result, the configuration of multiply damaged sites in DNA may comprise 3'-8-oxoG residues immediately next to a single-strand break. Consistent with these results, deletion of both *OGG1* coding for 8-oxoG-DNA glycosylase and *APN1* causes a nearly 46-fold synergistic increase in the spontaneous mutation rate and this enhanced mutagenesis is primarily due to G•C to T•A transversions.⁷⁸ Thus, Apn1 is involved in an Ogg1-independent repair pathway for 8-oxoG residues. Interestingly, the human major AP endonuclease, Ape1, also exhibits similar exonuclease activity towards DNA single-strand breaks containing 3'-end 8-oxoG residues,⁸² raising the possibility that this enzyme could participate in the prevention of mutations that would otherwise result from the incorporation of 8-oxodGTP. The observations that the *ogg1*^{-/-} null mice have no marked tumor predisposition^{83,84} point to redundancy in the repair mechanisms and a possible role of the AP endonucleases in preventing the accumulation of oxidized bases in the genome.

Nfo possesses an intrinsic 3'→5' exonuclease activity, however, pH, ionic strength and divalent cation requirements for this function versus the AP endonuclease activity are dramatically different.⁸⁵ It was proposed that the Nfo exonuclease activity is likely to follow AP site incision and may lead to resynthesis of DNA 5' to AP sites.⁸⁵ But, reconstitution of the *E. coli* BER pathway using cell-free extracts and purified enzymes (including Nfo) showed no detectable resynthesis of DNA 5' to the lesion sites.⁸⁶ Expression of the Nfo-H69A and Nfo-G149D mutants in a repair-deficient strain of *E. coli* complemented its hypersensitivity to alkylation but not to *t*-BuO₂H and bleomycin.^{5,67} Although bleomycin-treated DNA is cleaved more efficiently in vitro by Nfo than by Xth,⁸⁷ the nature of the specific types of DNA damage induced by bleomycin and *t*-BuO₂H whose repair in vivo requires Nfo have not yet been established. Nevertheless, it was shown that bleomycin attacks successively the two DNA strands to generate clustered DNA damage such as oxidized bases and/or AP sites located closely to breaks on the opposite strand.⁸⁸ Although, an oxidized base next to a single-strand break is highly resistant to the BER pathway,^{41,89} the excision of a bleomycin-induced damaged base situated two-three nucleotides away from the nick by a DNA glycosylase generates lethal double-strand breaks.⁹⁰

In this chapter, we propose a model where the AP endonuclease-catalyzed NIR coupled to a 3'→5' exonuclease activity initiates processing of bi-stranded clustered lesions without generating lethal double-strand breaks (Fig. 3). Ape1-catalyzed incision 5' to one of the damaged bases at a cluster (Fig. 3, step 1) is followed by 3'→5' exonuclease degradation which generates single-stranded gaps at the cluster site (step 2). In addition to high-fidelity replicative DNA polymerases, cells contain specialized DNA polymerases involved in translesion synthesis (TLS) and mutagenesis.⁹¹ Most of them belong to a new family of polymerases designated the Y-family, which have conserved sequences in the catalytic N-terminal half of the proteins. These polymerases have different efficiencies and specificities in vitro depending on the type of template damage. In eukaryotes, TLS requires post-translationally modified proliferating cell nuclear antigen (PCNA) and other PCNA-like factors such as the Rad9-Rad1-Hus1 (9-1-1) checkpoint complex which can recruit TLS-DNA polymerases to catalyse the incorporation of a small number of nucleotides directly opposite and downstream of the site of base damage.^{92,93} In the proposed model, exonucleolytic removal of the nucleotide 5' to the lesion in duplex DNA would leave lesions on the opposite strand either within or close to a single-stranded gap thereby preventing abortive DNA glycosylase-initiated excision repair (Fig. 3, step 2). The regions of single-stranded DNA generated downstream of a noncleaved lesion are expected to recruit modified PCNA or PCNA-like processivity factors in vivo (step 3).⁹⁴ These factors in turn would recruit TLS DNA polymerases which would then fill the single-stranded gap and bypass the template lesion (step 4). Removal of the remaining 5'-dangling base left after Ape1 incision would require strand displacement through continued DNA synthesis and cleavage of the dislodged 5'-strand by the flap-structure endonuclease 1 (FEN-1) stimulated either by the PCNA or by the 9-1-1 complex. Finally, DNA glycosylases will remove the remaining lesions from the restored duplex in the classic BER pathway. Thus, clusters might be handled by the

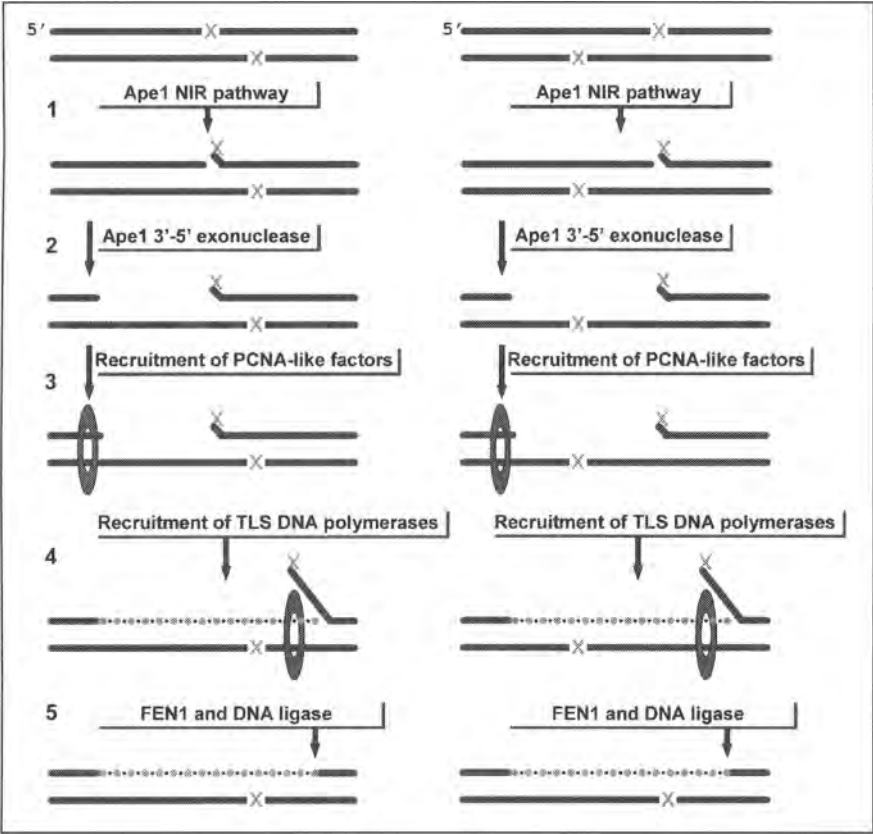


Figure 3. Schematic representation of the proposed model for processing of the bi-stranded clustered lesions.

coordinated intervention of NIR, exonuclease, TLS and BER pathways, defining a network of crosstalking proteins dedicated to preventing the formation of double-strand breaks.

Conclusions

The AP endonucleases Nfo, Apn1 and Ape1 are multifunctional DNA repair enzymes possessing AP endonuclease, 3'-repair diesterase, 3'→5' exonuclease and NIR activities. It was thought that these AP endonucleases mainly function in the BER pathway protecting cells from abasic sites and single-strand breaks with 3'-blocking groups induced either by DNA damaging agents or by DNA glycosylases.^{58,95} The AP endonuclease-initiated NIR works in concert with the BER pathway to cleanse genomic DNA of potentially mutagenic and lethal lesions.^{4,78} Recently we have demonstrated, using a genetic dissection of the BER and NIR functions of Nfo, that the drug sensitivity of *E. coli* correlates with the specific lack of NIR activity, thus strengthening the role of DNA glycosylase-independent repair pathway. In the present chapter, we propose a model where the AP endonuclease-catalyzed NIR coupled to 3'→5' exonuclease activity initiates processing of bi-stranded clustered lesions without generating lethal double-strand breaks. In conclusion, mechanistic understanding of the key steps in the repair of complex DNA lesions may lead to the identification of genetic factors associated with chronic diseases and therefore help to develop new prevention and therapeutic strategies.

Acknowledgements

The authors wish to thank Leela Daya-Grosjean for critical reading of the manuscript. This work was supported by the FP6 Euroatom Grant RISC-RAD FI6R-CT-2003-508842, Association pour la Recherche sur le Cancer, Electricité de France Contrat Radioprotection to M.K.S. S.C-P. was supported by a postdoctoral fellowship from the European Community.

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CHAPTER 5

OGG1:

From Structural Analysis to the Knockout Mouse

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Abstract

Among the four DNA bases, guanine, having the lowest redox potential, is the most susceptible to oxidation, and among the oxidized bases 7,8-dihydro-8-oxoguanine (8-oxoG) is certainly the lesion that has retained most attention over many years. This altered base can pair with A as well as C residues during replication. Eukaryotic cells use a specific DNA glycosylase, 8-oxoG DNA glycosylase (OGG1), to excise 8-oxoG from DNA, and repair-deficient cells are characterized by an increased G to T transversion frequency. It is essential that OGG1 has the ability to distinguish between 8-oxoG:C and 8-oxoG:A pairs and only removes 8-oxoG paired with C. This review will discuss the structural basis for OGG1 damage recognition and specificity as well as the literature on the biological consequences of OGG1 deficiency.

Introduction

Oxidation of DNA induced by reactive oxygen species can cause mutations, and more than 50 different modifications have been identified in cellular DNA, induced by γ -irradiation.¹ Such damage is ubiquitous in all living organisms and includes the highly mutagenic 8-oxoG lesion which, in the *syn* conformation, can mispair with A during DNA replication and cause GC to TA transversions.²

Repair processes for mutation avoidance at 8-oxoG lesions in genomic DNA are remarkably well conserved between bacteria and higher eukaryotes. *E. coli* have two DNA glycosylases—the Fpg enzyme which excises 8-oxoG paired with C, and the MutY enzyme which excises A opposite 8-oxoG^{3,4}—these enzymes work together to avoid mutations at 8-oxoG lesions.⁵ In mammalian cells the same functions are carried out by 8-oxoG DNA glycosylase (OGG1) and a MutY homologue (MYH), respectively (Fig. 1). OGG1 is currently among the best studied of a large group of DNA glycosylases that scan DNA for damaged bases, and initiate the highly conserved base excision repair (BER) pathway. Briefly, this pathway consists of two steps: excision of the damaged base by a DNA glycosylase and subsequent generation of an apurinic/aprimidinic (AP) site, followed by base replacement, catalyzed by the consecutive action of several other enzymes, among these APE1, DNA polymerase β (Pol β) and ligase I in humans.^{6–8}

The DNA repair glycosylases are broadly classified as monofunctional or bifunctional.^{9–11} Monofunctional glycosylases are hydrolases that use an activated water molecule to attack the glycosidic bond of the damaged nucleotide and produce an AP site in DNA. Bifunctional

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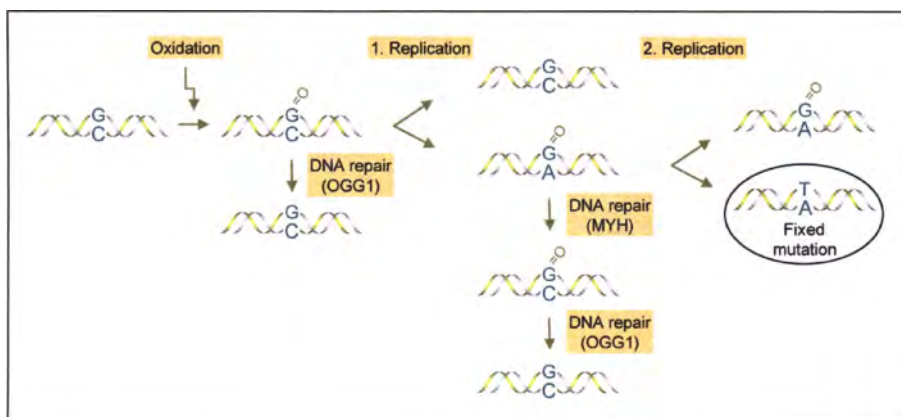


Figure 1. Repair, replication and mutagenesis at 8-oxoG lesions in DNA. A high proportion of 8-oxoG in DNA is induced following oxidative stress. This lesion is efficiently removed by OGG1. If left unrepaired, e.g., in OGG1 deficient cells, 8-oxoG can base pair with adenine (A) during replication. This is illustrated by the “1. replication” in the figure. Adenine base paired with 8-oxoG are removed by MYH, and the 8-oxoG:C base pair created is again a substrate for OGG1. Any 8-oxoG:A mispairs escaping repair will certainly lead to mutations as adenine will basepair with thymine (T) during the second round of replication.

glycosylases instead use an active site residue containing an activated amino group as a nucleophile. After expelling the damaged base, the reaction intermediate undergoes rearrangement to produce a ring-opened Schiff base that subsequently facilitates a base-catalyzed β -elimination reaction with scission of the sugar-phosphate backbone on the 3'-side of the damaged site (β -lyase activity). OGG1 has been classified as a bifunctional DNA *N*-glycosylase that catalyzes the release of oxidized purines from DNA, although the release of 8-oxoG is by far the most studied. The extensive investigations carried out with OGG1 include high resolution crystal structure, detailed biochemical analysis and the generation of *OGG1* targeted mutations in various species, as well as *OGG1* mutations in combination with several other mutated DNA repair genes. Recently, several comprehensive reviews on oxidative DNA damage and repair have been published.¹²⁻¹⁶ This chapter will discuss the OGG1 enzyme exclusively.

Cloning of *OGG1*

The *OGG1* gene was discovered in the mid 1990s when a mutator strain of *E. coli* (*fpg mutY*) was used to clone the *OGG1* gene from *S. cerevisiae* by transformation of a yeast DNA library and selecting clones with reduced spontaneous mutation frequencies.¹⁷ Such complementation assays had previously been shown to be incredibly valuable for the cross-species identification of DNA repair enzymes for alkylation damage.¹⁸⁻²⁰ The *OGG1* gene was demonstrated to encode a DNA glycosylase activity that excises 8-oxoG from DNA.^{17,21} Based on the homology between the *S. cerevisiae* and human enzyme, the release of the coding sequence for the human *OGG1* gene (*hOGG1*) encouraged cloning of the gene and characterization of the hOGG1 enzyme in eight laboratories at the same time.²²⁻²⁹ The similarity between the yeast and human proteins spans the whole of the sequence with an average 38% identity, but several regions that are as much as 60% identical can be found (Fig. 2). The OGG1 enzymes and *E. coli* Fpg are structurally unrelated and there is no indication of any homology.²³ Nevertheless, the activities of the enzymes are very similar, and in line with this, the eukaryotic enzymes can complement the *E. coli* *fpg* mutant. In the initial papers of the cloning of mammalian *OGG1*, several laboratories demonstrated that the mutator phenotype of *E. coli* *fpg/mutY* could be complemented by OGG1 also.^{22-24,26-28}

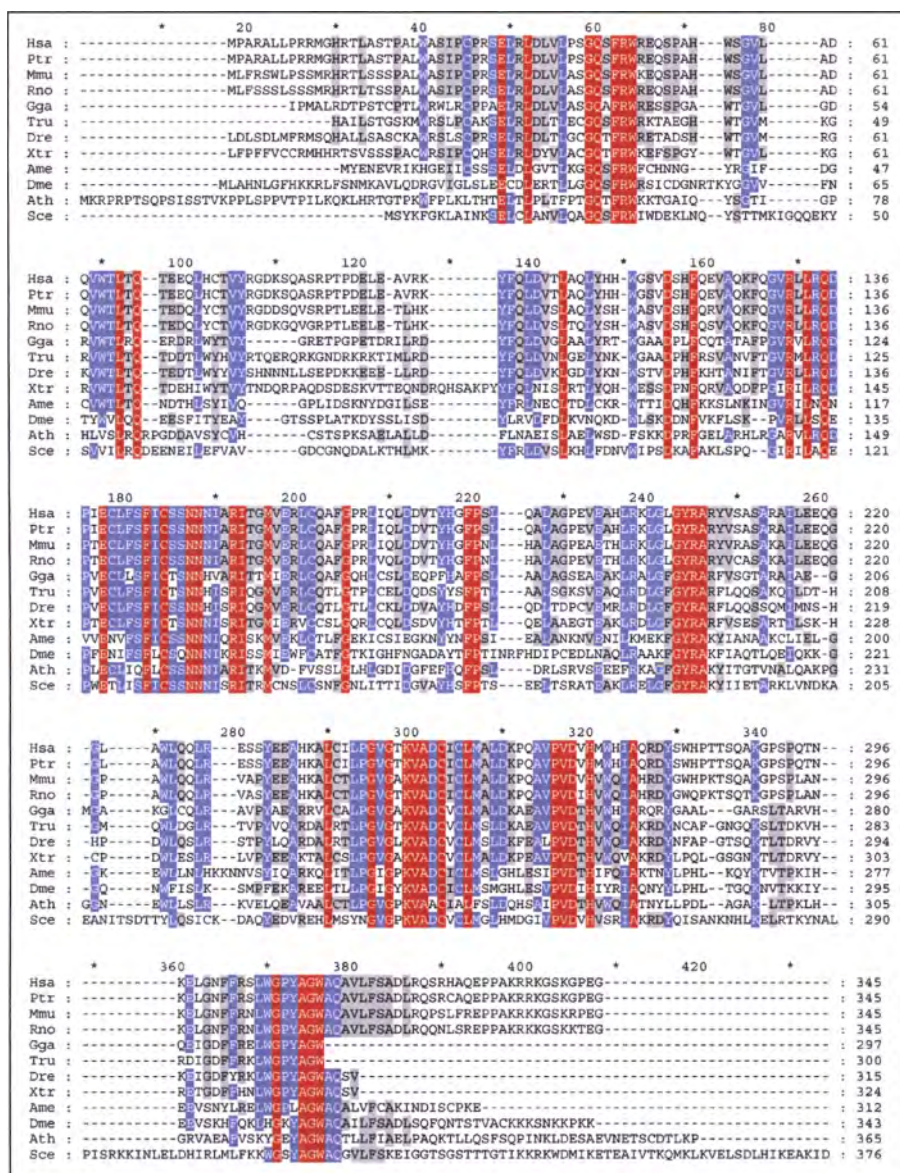


Figure 2. Multiple sequence alignment of selected OGG1 proteins. Residues conserved in at least 75% of the sequences are shown on a blue background, while residues conserved in at least half of the sequences are shown on a grey background. Absolutely conserved residues are shown on a red background. The source organism and the RefSeq⁹⁵ accession number or the ENSEMBL⁹⁶ identifier of the sequences are: *Homo sapiens* NP_002533 (Hsa), *Pan troglodytes* ENSPTRP00000043670 (Ptr), *Mus musculus* NP_035087 (Mmu), *Rattus norvegicus* NP_110497 (Rno), *Gallus gallus* ENSGALP_00000010744 (Gga), *Takifugu rubripes* SINFRUP_00000156793 (Tru), *Danio rerio* ENSDARP00000043181 (Dre), *Xenopus tropicalis* ENSXETP00000051069 (Xtr), *Apis mellifera* XP_394572 (Ame), *Drosophila melanogaster* NP_572499 (Dme), *Arabidopsis thaliana* NP_173590 (Ath) and *Saccharomyces cerevisiae* NP_013651 (Sce). The alignment was computed using MUSCLE⁹⁷ and drawn using the GeneDoc software (Nicholas and Nicholas, 1997, unpublished).

The cloning of the human *OGG1* gene also uncovered the existence of two splice variants, α -*hOGG1* and β -*hOGG1* with an open reading frame (ORF) coding for peptides of 345 and 424 amino acids, respectively.^{12,27} The β -*OGG1* gene structure appears to be absent in rodents. Later studies have identified several alternatively spliced forms of human OGG1 mRNAs, with the two variants previously mentioned being predominant.³⁰ These two alternative spliced forms are localized to the nucleus (α -*OGG1*) and mitochondria (β -*OGG1*).³⁰

Substrate Specificity of OGG1

OGG1 is a bifunctional DNA glycosylase with both *N*-glycosylase and β -lyase activities. The initial characterization of purified OGG1 from *S. cerevisiae* revealed an activity for release of 8-oxoG and 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyG) lesions (Fig. 3).¹⁷ These two lesions are also substrates for the mammalian OGG1 enzyme. In addition, OGG1 incises 7,8-dihydro-8-oxoadenine (8-oxoA) lesions.³¹⁻³³ Although OGG1 repairs all these lesions with similar efficiency, dependent upon the base on the opposite strand (discussed below), the vast majority of research has focused on 8-oxoG repair.³³ Nevertheless, one should have in mind that all three lesions are induced at significant rates by oxidative stress.³⁴ Also, 8-oxoA is as mutagenic as 8-oxoG and leads to A to G transitions and A to C transversions.^{35,36}

OGG1 Structure

The structure and reaction mechanism of hOGG1 has been elucidated by structural studies of the native protein by Bjørås and colleagues³⁷ and of hOGG1:DNA complexes in an impressive series of articles by Verdine and coworkers³⁸⁻⁴³—crystal structures of the preglycosylation/substrate recognition complex (K249Q and D258N mutants),^{39,42} the Schiff base intermediate (i.e., the borohydride trapped intermediate that differs from the imine only by the bond order of the C-N bond) with and without the expelled 8-oxoG in the active site

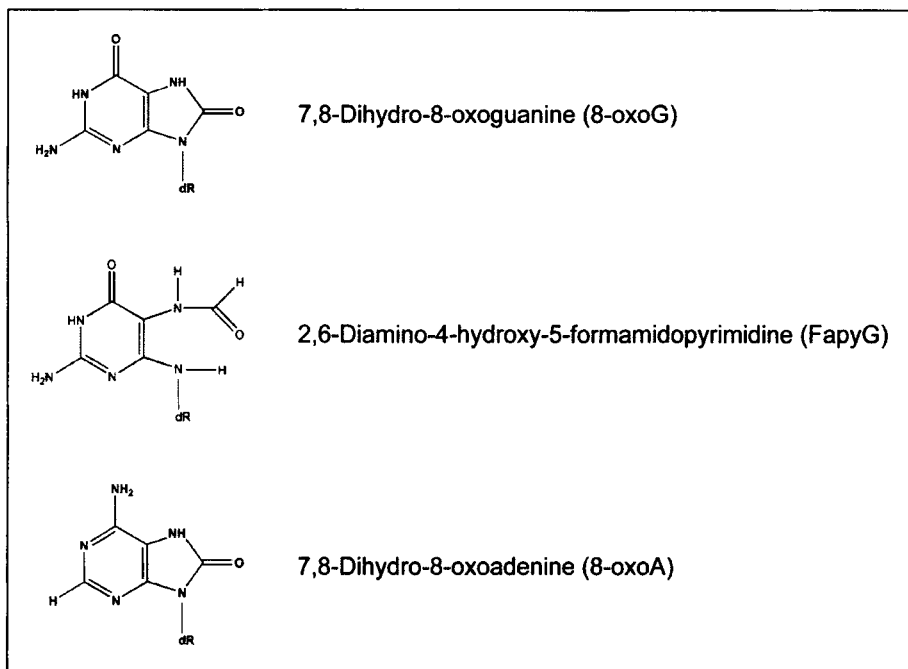


Figure 3. Base lesions removed by OGG1. The three quantitatively most important substrates for the OGG1 enzyme are illustrated.

pocket,⁴¹ and the post-reaction complex.⁴⁰ In addition, the Verdine group has published a structure of hOGG1 cross-bound to undamaged DNA which appears to be a good model of hOGG1 interrogating DNA for oxidation damage³⁸ and an atomic-force microscopy study of hOGG1 scanning DNA.⁴⁴

The hOGG1 protein belongs to a structural superfamily with an overall folding that is similar to for example *E. coli* AlkA, MutY and endonuclease III,³⁹ as well as the recently discovered archaeal AGOG proteins.⁴⁵ Members of this protein family are responsible for excision of a range of DNA bases that have been damaged by, for example, alkylation, deamination and oxidation. They are characterized by a common HhH-GPD motif—a helix-hairpin-helix (HhH) structural element^{46,47} followed C-terminally by a Gly/Pro-rich loop and an absolutely conserved Asp.^{21,39} The hOGG1 structure consists of two mainly α -helical domains that are common to all proteins of the Endo III superfamily; one of them containing the HhH motif. In addition, the protein contains an N-terminal domain also found in AlkA comprising an anti-parallel β -sheet (Fig. 4).

The crystal structure of the catalytic domain of hOGG1(K249Q) bound to 8-oxoG:C containing DNA reveals that hOGG1 binds DNA in the cleft between the two α -helical domains.³⁹ In the complex, the 8-oxoG base is fully extruded from the DNA duplex and inserted into a deep lesion recognition pocket, while the cytosine on the complementary strand remains intrahelical. There are only small local changes in the geometry in the active site region during the catalytic cascade, as revealed by the structure of the trapped intermediates and the end products.^{40,41} In the region of the 8-oxoG:C base pair, the DNA duplex is sharply bent ($\sim 70^\circ$) away from hOGG1 with a Ca^{2+} ion relieving the electrostatic repulsion between phosphate groups p^{-1} and p^1 (Figs. 4,5). The calcium ion might be replaced with magnesium in vivo.

Figure 5 shows a diagram of the hOGG1:(8-oxoG:C)-DNA interactions based on the published crystal structure.³⁹ The 8-oxoG base is deeply inserted into a pocket that appears to be able to exclude cytosine, adenine and thymine, as well as guanine. While there appears to be no direct interaction between hOGG1 and the carbonyl function at the C8 position of 8-oxoG, the Gly42 residue of the protein recognizes the other unique feature of 8-oxoG, its hydrogen at N7, by a hydrogen bond to the Gly42 backbone carbonyl group. This enables the protein to recognize also FapyG and 8-oxoA which contains a hydrogen bond donor in the same position. Gly42 is the only residue of the AlkA/OGG1-type N-terminal domain that is directly bonded to DNA. The HhH motif (residues approximately 235 to 258) is mainly in contact with the DNA backbone 3' to the 8-oxoG lesion (Fig. 5).

On the complementary strand, cytosine is not extruded from the DNA duplex, but is nevertheless unstacked from its neighboring DNA bases due to the strong bending of the DNA helix and the insertion of hOGG1 residues 5' (Tyr203) and 3' (Arg154) to the base. The two residues Arg154 and Arg204 establish strong bidentate hydrogen binding with the two hydrogen bond acceptors on cytosine, O2 and N3. None of the other DNA bases have two close-lying hydrogen bond acceptors available for this kind of interaction, and this explains the specificity of hOGG1 for 8-oxoG lesions complementary to cytosine.³⁹ Interestingly, an hOGG1(R154H) mutant found in certain human gastric cancers⁴⁸ readily performs promutagenic repair of 8-oxoG:A due to its inability to discriminate cytosine from other bases complementary to 8-oxoG.³⁹

A comparison of the crystal structure of the free protein and the hOGG1:DNA complexes shows the overall conformations to be similar except for fairly small changes in backbone and side-chain conformations in the binding and active site region.³⁷ Interestingly, the active site pocket is closed off in the free enzyme, and it has been proposed that the three residues Phe319, Gln315 and His270 might be acting as a single functional entity switching between an open (complex) and closed (free enzyme) state.³⁷ It appears that the full catalytic machinery of hOGG1 is not assembled until the damaged DNA base has been fully inserted into the active site pocket.^{37,43}

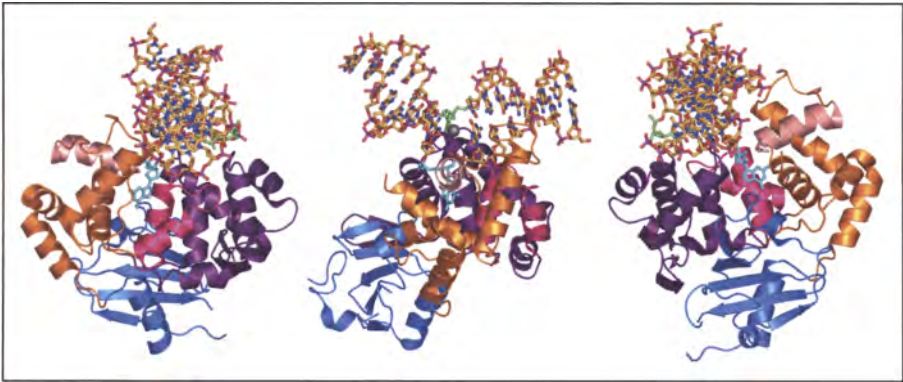


Figure 4. Please see figure legend on next page.

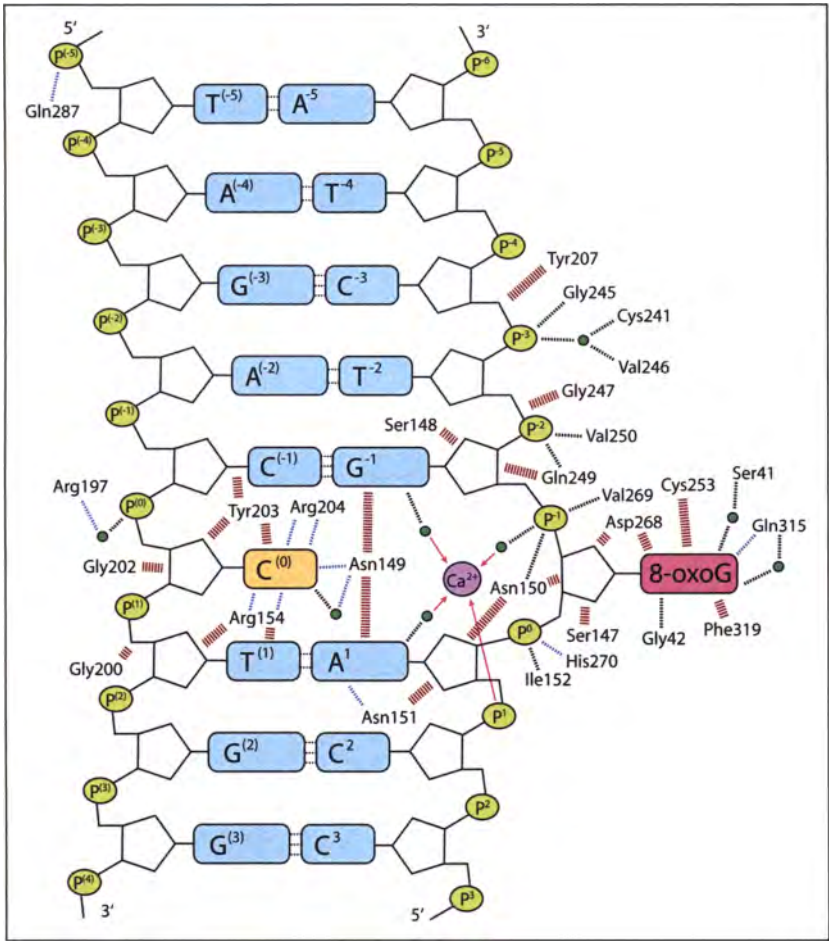


Figure 5. Please see figure legend on next page.

Figure 4, viewed on previous page. Structure of hOGG1 bound to 8-oxoG:C-containing DNA based on the Protein Data Bank (PDB) structure 1EBM of Verdine and coworkers³⁹ with the two α -helical domains (orange/salmon and purple/pink) and the anti-parallel β -sheet N-terminal domain (blue) viewed approximately along the axis of the duplex DNA from the "front" (left) and "rear" side (right) and perpendicularly to this axis (middle). The extruded 8-oxoG residue is shown in cyan and the complementary strand C in light green. The active site helix capped N-terminally by Asp268 is shown in salmon, while the HhH motif is shown in pink. The Ca^{2+} ion is shown as a grey sphere.

Figure 5, viewed on previous page. Schematic representation of the interactions between hOGG1(K249Q) and 8-oxoG:C-containing DNA based on the PDB structure 1EBM of Verdine and coworkers.³⁹ In the native protein Gln249 is replaced by Lys. All hydrogen bonds are given as black broken lines, except direct hydrogen bonds between hOGG1 amino acid side chain atoms and DNA which are given in blue. Nonbonded/van der Waals contacts are given as thick, red, broken lines and the coordinative interactions of the Ca^{2+} ion as red arrows. Water molecules visible in the crystal structure that are mediating hydrogen bonding interactions are given as small green circles.

OGG1 Catalytic Mechanism

The OGG1 proteins have both glycosylase and β -lyase activities, but a number of studies have shown the glycosylase activity to be significantly higher than the lyase activity.^{24,32,49,50} It has also been found that the two enzymatic steps in general are affected differently by various cofactors, e.g., physiological concentrations of magnesium ions⁵¹ or the presence of other proteins of the BER pathway such as APE1 and XRCC1.^{8,49,50,52,53} The excised 8-oxoG in the catalytic pocket also inhibits the strand incision reaction step⁵¹ while the presence of other guanine analogs in the catalytic pocket increases the lyase activity.^{41,51} Based on the above results, it has been argued that OGG1 represents a type of glycosylase with fairly weak and *uncoupled* lyase activity.^{8,24,32,49-51,53} It has also been argued that the lyase activity of OGG1 might be completely bypassed *in vivo*, with the AP-DNA being passed directly on to an AP endonuclease.^{49,51,53} This is consistent with the work of Allinson et al⁵⁴ which demonstrates that the 5'-deoxyribosephosphate lyase activity of Pol β is essential for 8-oxoG repair and that it is mainly Pol β that incise the DNA backbone 3' to the 8-oxoG lesion. A number of alternative reaction mechanisms have been proposed and partially tested, but at present there seems to be no consensus on the details of the catalytic mechanism.^{6,37,39-42,55} In addition, recent studies have shown the properties of hOGG1 to be altered by post-translational modifications such as phosphorylation *in vivo*, adding another level of complexity to these questions.⁵⁶⁻⁵⁸

A number of OGG1 mutants have been generated in biochemical investigations that give important clues about the catalytic mechanism of OGG1. Among these mutants hOGG1(K249Q) has no catalytic activity, demonstrating the importance of Lys249 for the glycosylase activity.^{39,59} This amino acid is located in the catalytic pocket and is the obvious candidate to act as a nucleophile on C1' of the glycosidic bond and expel 8-oxoG. Reaction mechanisms for this glycosylase step have been proposed where this substitution reaction occurs without activation of the leaving group, as well as after activation by protonation on O8 of 8-oxoG or O4' of the deoxyribose.^{6,32} However, in the crystal structure of hOGG1(D268N):8-oxoG-DNA, Lys249 is not well positioned for nucleophilic attack on C1'. It was consequently suggested that hOGG1 employs a dissociative $\text{S}_{\text{N}}1$ -like mechanism for the glycosylase step, instead of a direct $\text{S}_{\text{N}}2$ -type nucleophilic substitution.⁴² Theoretical calculations by Schyman et al⁵⁵ appear to lend support to this hypothesis by showing that an $\text{S}_{\text{N}}1$ -type mechanism, with the protonated ϵ -amino group of Lys249 acting to stabilize the developing negative charge on the 'leaving' 8-oxoG base gives a lower calculated reaction energy profile than some alternative mechanisms.

The role of Asp268, the only amino acid in the active site region that is absolutely conserved, has not been finally determined, but work by Verdine and coworkers has determined

the effect of mutating this residue and replacing Asp268 by Asn, Glu, and Gln.⁴² The hOGG1(D268N) mutant binds DNA as strongly as the wild type protein, but has very little enzymatic activity, this being an indication of the importance of the acid function presence in the active site. On the other hand Asp268 is also important for the stability of the hOGG1 protein fold⁴² with this active site Asp being involved in N-terminal capping of an α -helix that is bordering the active site pocket (Fig. 4). All three Asp268 mutants show significantly reduced stability compared with the wild type enzyme. Interestingly, the D268E and D268Q mutants have nearly wild type catalytic activity at 25°C, a temperature at which they are stable. In view of the complete lack of activity for D268N, the authors suggest that the activity of D268Q might be due to hOGG1(D268Q) acting as a monofunctional glycosylase with an activated water molecule being the nucleophile in the glycosylase step.⁴²

Boiteux and coworkers have investigated the importance of the residues Phe319, Gln315, and His270 lining the lesion recognition pocket of hOGG1.⁶⁰ They find that H270A, H270L, and F319A mutants have very limited glycosylase activity, most likely due to their reduced 8-oxoG-DNA binding affinity. The Gln315 residue appears to be less important for the binding properties and catalytic activity. Hashiguchi et al⁶¹ generated a range of hOGG1 mutants and showed that the correct folding of the C-terminal α -helix (residues 313-322) is essential for the glycosylase activity of hOGG1. Interestingly, this α -helix is not complete in mitochondrial β -hOGG1, which consequently lacks glycosylase activity. This suggests a different role for β -hOGG1 in mitochondria.

Whilst there is still no general agreement on the mechanism by which hOGG1 removes 8-oxoG from DNA, and the way in which and how efficiently, if at all, it cleaves the DNA backbone in vivo, further experiments are likely to clarify this issue within the next few years. The possibility remains open that the various forms of hOGG1—iso-forms and/or products of post-translational modifications—employ reaction mechanisms that differ depending on the environment, pathway, and other factors.

OGG1 Mutants

Five years ago, two independent studies described the generation and initial characterization of mice targeted for the *OGG1* gene.^{62,63} Mice lacking the OGG1 enzyme were found to have physically normal appearance. On the other hand, the amount of 8-oxoG in tissues accumulated to abnormal levels, and this accumulation was shown to be age related and tissue specific.^{63,64} The results suggested that the consequences of OGG1 depletion were restricted to slowly proliferating tissues with high oxygen metabolism, such as liver.⁶⁴ The spontaneous mutation frequency of the slowly proliferating liver was found to increase 2 - 3 fold following *OGG1* inactivation,^{62,63} whereas no increase was observed in rapidly proliferating tissues.⁶² The increased mutation load in the liver was almost exclusively due to GC to TA transversions believed to originate from 8-oxoG mispairing with A during replication. This is illustrated in Figure 6. Furthermore, OGG1 knockout mice have normal somatic hypermutation of Ig genes.⁶⁵

A third study⁶⁶ found that lung adenoma/carcinoma spontaneously developed in *OGG1* knockout mice analyzed 1.5 years after birth. In order to weaken the cellular defense against oxidative DNA damage further, they also generated *OGG1/Mth1* (mutT homologue) double knockout mice. MTH1 hydrolyzes 8-oxo-dGTP to 8-oxo-dGMP, and this enzyme is responsible for preventing misincorporation of 8-oxoguanine into DNA. Surprisingly, no tumors were identified in double knockout mice.⁶⁶

Following the initial publications of the *OGG1* knockout mouse⁶² a review paper by Boiteux and coworkers¹² concluded: "Extensive analysis of *OGG1* null animals and construction of animals deficient in more than one repair mechanism is required to assess the biological impact of 8-oxoG in mammalian cells". This has indeed been shown to be correct. Another DNA glycosylase, MYH in mammals and MutY in *E. coli*, removes adenine from

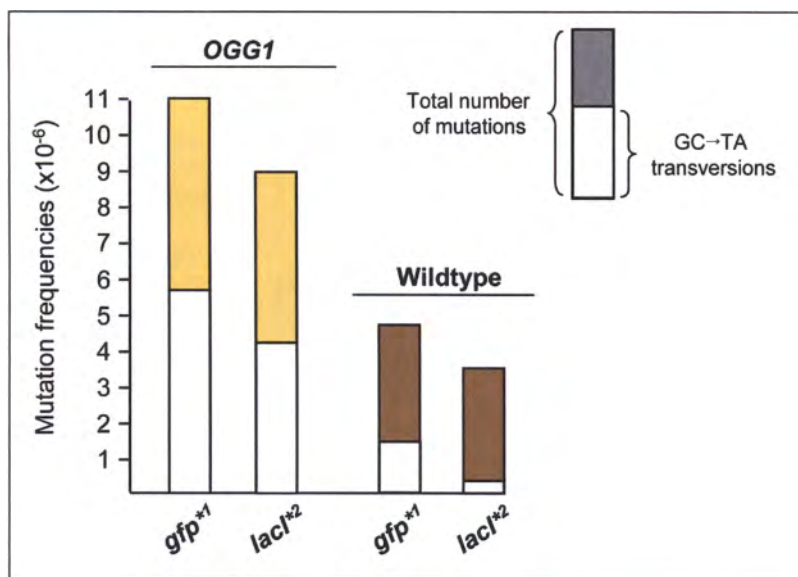


Figure 6. Spontaneous mutagenesis in reporter genes in liver DNA from *OGG1* knockout mice. Two different studies have evaluated the spontaneous mutation frequency in *OGG1* deficient mice in vivo. Mutation analyses were carried out by breeding the *OGG1* knockout mice to *gfp* or *lacI* transgenic mice. The chart presents the overall mutations observed, with the proportion of GC→TA transversions illustrated as the white part of the poles. Mutation analysis of the *OGG1 gfp* transgene (^{*1}) are from Minowa et al.⁶³ and the *OGG1 lacI* transgene (^{*2}) analysis from Klungland et al.⁶²

8-oxoG:A mismatches, which results from erroneous incorporation of adenine opposite 8-oxoG during replication (see Introduction). Therefore, simultaneous deletions of both glycosylases would most likely cause a considerable increase in the GC to TA transversion frequency. A strong mutator phenotype of this type was identified in *E. coli* (*mutM mutY*) several years ago.⁶⁷ A recent comprehensive study addressed if this was also the case in mice.⁶⁸ The generation of *OGG1* and *Myh* double knockout mice^{62,68} demonstrated that combined deficiencies of these genes predisposed 2/3 of the mice to tumors, establishing an obvious link between BER deficiency and tumorigenesis.⁶⁸ One remarkable similarity between the *Myh* related tumors in humans and *Myh/OGG1* related tumors in mice is that subsequent analysis identified unique G to T mutations in codon 12, GGT, of *K-ras*.⁶⁸⁻⁷⁰ This might reveal a distinctive common pathway of tumorigenesis through *K-ras* activation following DNA oxidation in a repair deficient background. The mice described thus provide a unique model for studying the mechanisms of oxidatively damaged DNA in tumorigenesis.

The *OGG1* knockout mouse has also been widely utilized to define the role of *OGG1* for mitochondrial DNA repair,⁷¹ transcription-coupled repair,⁷² its cross-talk with the Cockayne syndrome B protein⁷³⁻⁷⁵ and also its role in the presence of 8-oxoG in the urine.⁷⁶

Several single nucleotide polymorphisms of *OGG1*, with resultant amino acid substitutions of the protein, have been registered in the human population and Ser326Cys is the most common polymorphism.⁷⁷ The potential risk of cancer associated with such polymorphic *OGG1* has been addressed in numerous studies. However, although it is proposed that *OGG1* Ser326Cys might be a risk allele,^{78,79} there is no clear evidence of this⁸⁰⁻⁸² and conflicting results have been obtained for the enzymatic activity of mutated hOGG1.⁸³

Backup Activities for OGG1

Fapy lesions in DNA are substrates of both purified Nth (endonuclease III) and OGG1, and this property is shared by the yeast and mammalian enzymes.^{17,24,84-86} In accordance with these in vitro data, it was shown that *OGG1* knockout mouse had reduced, but still significant, capacity for repair of FapyG lesions.⁶² Thus, it seems that OGG1 and Nth both repair FapyG lesions with high efficiency. In addition, this is also an activity for the recently identified NEIL1 (Nei-like) DNA glycosylase.⁸⁷ Thus, at least three enzymes repair the same lesion.

The situation is less clear for the redundancy of enzymes repairing 8-oxoG and 8-oxoA lesions. It was shown, many years ago, that purified alkylbase DNA glycosylase, ANPG, could repair 8-oxoG in vitro.⁸⁸ However, mice carrying a targeted deletion of the gene encoding ANPG had normal repair of 8-oxoG.⁸⁹ The *OGG1* knockout mice generated were characterized for the capacity to remove 8-oxoG lesions from their genomic DNA in vivo and by extracts from cells and tissues to remove 8-oxoG from synthetic oligonucleotides. The results clearly demonstrated that OGG1 was the major activity for 8-oxoG removal in liver extract, and that the activity was gene dosage dependent.^{62,63} Although the activity was never totally zero in OGG1 deficient extracts, the residual activity of unknown origin in homozygotes was at most less than 5% of that for wild-type mice. The opposite base dependence of the OGG1 enzyme in cellular extracts was as discussed above. The substrate specificity of nicking activity in liver extracts was also determined. Kinetic analysis in vivo gave the same result, e.g., a slow, but significant, removal of 8-oxoG in *OGG1* knockout cells.⁶² Recently, several "Nei-like" DNA glycosylases were identified in the human genome.^{87,90,91} Two of these, NEIL1 and NEIL2, are able to incise DNA at 8-oxoG lesions.^{87,92} With respect to this it is very exciting that these DNA glycosylases have a unique preference for excising lesions from a bubble structure, whereas OGG1 is active only with duplex DNA.⁹²

OGG1 Orthologs

Human OGG1 has orthologs in other mammals, including chimpanzee, dog, cow, mouse, rat, chicken and opossum. It is also present in fish (*Tetraodon nigroviridis*, *Takifugu rubripes*, *Danio rerio*), frogs (*Xenopus tropicalis* and *X. laevis*), insects (*Drosophila melanogaster*, *Apis mellifera*, *Anopheles gambiae*), plants (*Arabidopsis thaliana*, *Oryza sativa*), yeasts (e.g., *Saccharomyces cerevisiae*, *Neurospora crassa*), parasites (e.g., *Plasmodium berghei*, *Cryptosporidium hominis*, *Trypanosoma brucei*) and other eukaryotes, as well as a few eubacteria (e.g., *Aquifex aeolicus*) and archaea (e.g., *Archaeoglobus fulgidus*). OGG1 seems to be very widely distributed among eukaryotes with a notable exception of *C. elegans*. Interestingly, several other widespread DNA glycosylases also seem to be lacking in *C. elegans*. A multiple sequence alignment of selected OGG1 proteins is shown in Figure 2.

Conclusions

OGG1 is one of the best studied DNA repair enzymes and certainly the major repair activity for removal of 8-oxoG from DNA. Nevertheless, *OGG1* mutants in yeast and mice display only a moderately elevated mutation frequency, with no obvious phenotype. In mice, the combined *OGG1/Myh* deficiency causes a remarkably high incidence of cancer.⁶⁸ In yeast, the collaborative panel of repair strategies at the potentially mutagenic 8-oxoG lesion include BER, nucleotide excision repair, mismatch repair as well as damage bypass via translesion synthesis.⁹³ It must be expected that mammalian cells contain several, as yet unidentified, enzymes for mutation avoidance and some candidates have been characterized in vitro.^{87,94} Future studies should include the combined deficiency of such enzymes and the *OGG1* mutant mouse. A better understanding for the detailed catalytic mechanisms and possible interacting proteins is also required.

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CHAPTER 6

Processing of 3'-End Modified DNA Strand Breaks Induced by Oxidative Damage

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Abstract

In living cells, the DNA molecule is subject to attack from reactive oxygen species generated as the result of endogenous oxidative metabolism and exogenous factors, such as ionising radiation. Reactive oxygen species can produce a variety of DNA lesions, including DNA single strand breaks containing modified 3'-ends that are a threat to cellular genomic integrity. However, the cell is equipped with multiple repair mechanisms that are able to efficiently remove the lesion followed by subsequent repair of the DNA strand break. The majority of small base damages in DNA are repaired by proteins of the base excision repair pathway that involves removal of the damaged base by a DNA glycosylase, incision of the AP site produced by AP endonuclease and gap filling and ligation by DNA polymerase β and DNA ligase III α -XRCC1 complex, respectively. However, the repair of DNA single strand breaks containing 3'-end modifications may require a different subset of enzymes due to the different complexity of the damage. In this review, we summarise the proteins currently identified as playing a major role in the repair of DNA single strand breaks containing 3'-end lesions.

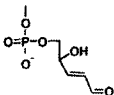
Introduction

A plethora of DNA lesions occur daily in living cells through oxidative stress caused endogenously, by oxidative metabolism, or exogenously by, for example, ionising radiation. The majority of the DNA damage produced by these processes is induced largely through generation of reactive oxygen species (ROS), such as superoxide ($O_2^{\cdot-}$) and the highly reactive hydroxyl radical ($\cdot OH$), that attack DNA. ROS can induce DNA single strand breaks (SSBs), base hydrolysis resulting in the formation of apurinic/apyrimidinic (AP; abasic) sites and oxidative DNA base damage, such as 8-oxoguanine or thymine glycol, that are potentially deleterious to the cell. Furthermore, ionising radiation can introduce these lesions in close proximity to each other on DNA as so called "clustered DNA damages".¹ ROS can also cause oxidative damage to the sugar moiety of DNA that can result in DNA fragmentation and the subsequent generation of SSBs containing damaged 5'- and/or 3'-ends.^{2,3} For example, terminal 3'-blocking groups containing 3'-phosphate or 3'-phosphoglycolate ends are a major product of ionising radiation.⁴ This wide variety of "damaged" SSBs must be converted to the conventional 3'-OH and 5'-phosphate nick before the DNA ends are joined by DNA ligase prior to DNA replication to prevent replication fork collapse that may result in loss or change of genetic information, which in turn may cause genome instability and enhance the progression of human diseases.

In this chapter we will discuss the human enzymes and mechanisms involved in repair of "damaged" SSBs.

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Table 1. Structures of the major 3'-end modifications

Structure	Name	Formation
	3'- α,β -unsaturated aldehyde	β -elimination by DNA glycosylase (e.g., OGG1, NTH1)
	3'-phosphoglycolate	Ionising radiation Bleomycin
	3'-phosphate	Ionising radiation β,δ -elimination by DNA glycosylase (e.g., NEIL1)

Formation of “Damaged” DNA 3'-Ends

Damaged DNA 3'-ends may arise as a result of direct chemical modification during SSB formation or during enzymatic processing of DNA damage. Endogenous oxidative metabolism and exogenous factors, such as ionising radiation, in addition to producing oxidative DNA damage, base modifications and AP sites, can also induce SSBs with modified 5'-and/or 3'-ends through the action of ROS. Indeed, oxidative damage to the sugar moiety of DNA can result in hydrogen abstraction from either the 1' or 4' carbons of deoxyribose that causes chemical rearrangements.³ This can lead to the subsequent generation of SSBs containing 3'-end phosphate or phosphoglycolate, respectively (Table 1). Since ionising radiation energy is deposited in small volumes of nanometre dimensions, this may result in ROS produced at high local concentrations.⁵ This in turn leads to the formation of multiply damaged sites in DNA that may include 3'-modified bases immediately next to a SSB. Furthermore, endogenous ROS can also induce oxidative DNA damage, such as 8-oxoguanine, directly or they can oxidize nucleotide triphosphates that can be later incorporated into DNA.⁶ However, both direct guanine oxidation by ROS and misincorporation of 8-oxodGTP by a DNA polymerase lacking proofreading activity can result in SSBs with 3'-terminal damaged bases.^{7,8} Although ionising radiation is a major contributor to the formation of damaged 3'-ends, antitumour drugs, such as bleomycin and neocarzinostatin, can also generate SSBs containing 3'-phosphoglycolate and 3'-phosphate, respectively.⁹

Base excision repair intermediates are the other major source of 3'-end damaged SSBs. The bifunctional glycosylases 8-oxoguanine DNA glycosylase (OGG1) and the endonuclease III homologue (NTH1) have an associated β -lyase activity that cleaves an abasic site on the 3'-side of the phosphodiester backbone to generate a 3'- α,β -unsaturated aldehyde. Therefore these lesions are formed during endogenous DNA damage processing. The formation of 3'-phosphate lesions is also apparent during base excision repair by the endonuclease VIII-like (NEIL) proteins that perform β,δ -elimination.¹⁰ Additionally, SSBs containing 3'-phosphate can be generated by tyrosyl-DNA phosphodiesterase (Tdp1) cleavage of 3'-phosphoglycolate since, although Tdp1 is mainly involved in cleavage of topoisomerase I-DNA adducts, it also has activity towards 3'-phosphoglycolates.¹¹

Repair of DNA Single Strand Breaks Induced by the Base Excision Repair Pathway

Poly(ADP)-ribose polymerase-1 (PARP-1) has a very high affinity for strand breaks and most probably binds to SSBs before any other repair proteins.¹² Binding of PARP-1 to strand breaks stimulates formation of poly(ADP)-ribose polymers and dissociation of PARP-1 from

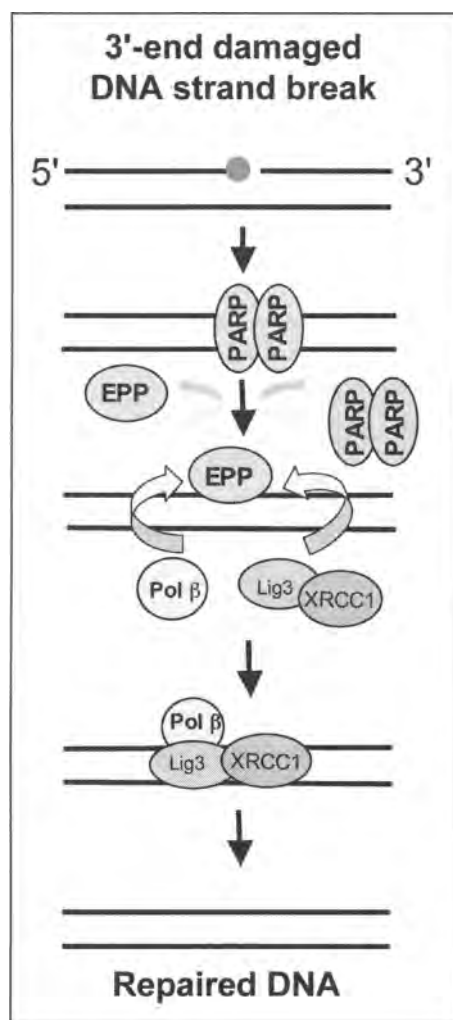


Figure 1. Model for repair of DNA strand breaks containing damaged 3'-ends. DNA strand breaks containing damaged 3'-ends are recognised by the corresponding End Processing Protein (EPP) that converts the modified 3'-ends into conventional 3'-hydroxyl ends and further recruits Pol β and XRCC1-DNA ligase III α to accomplish repair. Among the known EPPs are APE1, PNK and Tdp1 that recognise and process 3'-ends containing phosphoglycolate and phosphate groups.

the DNA.¹² The role of PARP-1 binding in SSB repair is unclear, since the rate of SSB repair,^{13,14} is not affected in PARP-deficient cells. After PARP-1 dissociation, repair proceeds by direct ligation of the frank DNA SSB, or may include an accompanying “cleaning and filling” of the strand break by an end processing protein (EPP) and DNA polymerase β , before ligation by DNA ligase III α -XRCC1 (X-ray cross complementing protein 1) complex (Fig. 1).¹⁵⁻¹⁷ XRCC1 protein plays a pivotal role in SSB repair by orchestrating the repair process through a series of protein-protein interactions and XRCC1 deficiency results in severe sensitivity to DNA damaging agents and genome instability (reviewed in ref. 18). However, although it is suggested that XRCC1 binds to SSBs at the early stages of repair and functions as a scaffold protein,¹⁸ the exact mechanism coordinating repair of SSBs is still obscure.

Table 2. Summary of major proteins involved in the processing of 3'-end modified DNA strand breaks

Protein	MW	End Processing Activity
AP endonuclease-1 (APE)	35.4 kDa	3'-phosphoglycolate ³¹⁻³⁸ 3'-phosphate ^{28,29} 3'-8-oxoG ⁴⁰ 3'- α,β -unsaturated aldehyde ³⁰ 3'-5'-exonuclease ³⁹
Polynucleotide kinase (PNK)	57.1 kDa	5'-kinase ^{30,42,43} 3'-phosphate
Tyrosyl-phosphodiesterase-1 (Tdp1)	68.4 kDa	3'-phosphoglycolate ^{11,51}

The 3'-blocking lesions that are formed during endogenous oxidative metabolism and from exogenous agents, such as ionising radiation, must be processed to generate 3'-hydroxyl and 5'-phosphate termini prior to gap filling by DNA polymerase β and ligation by DNA ligase III α -XRCC1. This entails an important role of end-processing enzymes in SSB repair and genome stability. To date, several 3'-end processors have been identified (Table 2), namely AP endonuclease 1, polynucleotide kinase and tyrosyl-DNA phosphodiesterase 1 that play a major role in repair of damaged SSBs.

AP Endonuclease 1 (APE1)

APE1 is the major AP endonuclease in human cells involved in the incision of abasic sites in DNA and thus forms an essential component of the base excision repair (BER) pathway. The APE1 protein is 318 amino acids in length and approximately 35 kDa with two distinct domains: an N-terminal domain that is essential for redox activity and contains a nuclear localisation sequence and a C-terminal region containing the endonuclease activity.^{19,20} APE1 is known to be absolutely required for cellular survival with deletion of APE1 in mice proving to be embryonically lethal,²¹⁻²³ and stable cell lines lacking the protein have not been established, again pointing to a critical role for APE1. It was demonstrated that in HeLa,²⁴ rodent,²⁵ and human glioma cells,²⁶ APE1 can be transiently elevated in response to oxidative DNA damage. Enhanced resistance to alkylating agents is also observed, consistent with increased AP endonuclease activity.^{24,26} More recently, downregulation of APE1 by RNA interference (RNAi) revealed that AP endonuclease activity is essential for cell viability and plays a central role in endogenous DNA damage processing.²⁷

APE1 is a multifunctional enzyme, displaying, in addition to AP endonuclease activity, 3'-phosphatase, 3'-phosphodiesterase and 3'- to 5'-exonuclease activities specific for the 3'-termini of internal nicks and gaps in DNA.²⁸ Unlike the bacterial and yeast AP endonucleases, which exhibit nearly equal endonuclease and 3'-phosphatase activities, the activity of human APE1 on 3'-phosphate containing substrates is reported to be ~200-fold lower than its endonuclease activity,^{28,29} and possibly suggests an alternative repair mechanism for this substrate. In agreement with this, a comparison of kinetic parameters indicates that the activity of polynucleotide kinase (PNK) against 3'-phosphate lesions supersedes APE1.³⁰ The phosphodiesterase activity of APE1 has been observed on a 3'-phosphoglycolate-containing substrate using both endogenous and recombinant sources of protein.³¹⁻³⁴ APE1 was shown to catalyse the release of a 3'-phosphoglycolate blocking group as free phosphoglycolic acid,³⁵⁻³⁷ and it has been further identified as the major human enzyme involved in the excision of 3'-phosphoglycolate, in a magnesium dependent manner.³⁸ This activity shows a preference for a one nucleotide gap substrate with 3'-phosphoglycolate termini over substrates containing 3'-phosphoglycolate on a 5'-protruding end or within blunt ended oligonucleotide duplexes.³⁷

The 3'- to 5'-exonuclease activity of APE1 on mismatched DNA bases at the 3'-termini of nicked or gapped DNA substrates is very low and requires an acidic environment.³⁹ Although it was suggested that this activity may be important as a mechanism for increasing fidelity during BER by correcting errors made by DNA polymerase β , this hypothesis needs more proof. However, it was recently demonstrated that APE1 excises 3'-8-oxoguanine residues present within a SSB.⁴⁰ This mechanism of 3'-end processing was base damage specific as the level of excision of a mismatched base pair was negligible in comparison, indicating that the 3'- to 5'-exonuclease activity of APE1 is relatively weak compared to its 3'-phosphodiesterase activity. Therefore APE1 may act as a nonspecific repair enzyme for "cleaning" 3'-blocked DNA ends. To this end, it is also known that APE1 excises the 3'- α,β -unsaturated aldehyde generated by bifunctional glycosylases, such as OGG1 and NTH1.³⁰

Interestingly, APE1 has been shown to interact with XRCC1,⁴¹ although the physiological significance of this interaction remains unclear since the first protein that is recruited to the incised AP site is PARP-1, rather than XRCC1.¹² However we have recently demonstrated that APE1 is required for the recruitment of XRCC1-DNA ligase III α heterodimer to a 3'-phosphoglycolate containing DNA strand break (Parsons et al 2005, FEBS J, in press) suggesting that the APE1-XRCC1 interaction may be important for the recruitment process.

Polynucleotide Kinase (PNK)

It is known that bacterial AP endonucleases contain efficient activity to remove 3'-phosphate lesions, however human APE1 has a very low 3'-phosphatase activity suggesting that a different protein may be involved in the repair of this lesion in mammalian cells. Human PNK, a homologue of T4-PNK, has been cloned and characterised as a human DNA 5'-kinase and 3'-phosphatase that is the principal enzyme involved in the restoration of 5'-phosphate and 3'-hydroxyl ends at SSBs.^{42,43} The human PNK protein is 521 amino acids in length and approximately 57 kDa in size and recently the crystal structure of mouse PNK has been resolved.⁴⁴ In addition to its catalytic kinase and phosphatase domains, it contains an N-terminal forkhead-associated (FHA) domain that mediates protein-protein interactions. PNK was thought to play a role in the repair of SSBs caused by oxidative DNA damage, as it has been shown to complement the hypersensitivity of AP endonuclease deficient bacteria.⁴² Furthermore, *S. pombe* cells lacking PNK were sensitive to camptothecin and ionising radiation.⁴⁵ Subsequently, in human cells, the mechanism of repair of oxidative strand breaks by PNK has been revealed by the interaction with components of the BER pathway, namely XRCC1, DNA ligase III α and DNA polymerase β .⁴⁶ In this study, it was found that XRCC1 can stimulate both the 3'-phosphatase and 5'-kinase activities of PNK and therefore accelerate DNA SSB repair. We have also recently demonstrated that PNK is required for the recruitment of XRCC1-DNA ligase III α heterodimer to a 3'-phosphate containing DNA strand break (Parsons et al 2005, FEBS J, in press).

PNK has also been implicated to function in double strand break repair by nonhomologous end joining by interaction with XRCC4,^{47,48} and recently, this important role for PNK in single and double strand break repair was revealed by downregulation of human PNK by small interfering RNA silencing technology.⁴⁹ Using this approach, a reduction in the levels of endogenous PNK enhanced the spontaneous mutation frequency and caused an elevated sensitivity of the cells to genotoxic agents, including γ -radiation and UV radiation. Furthermore, the repair of radiation induced DNA strand breaks was observed to be slower in the absence of PNK and overall this study demonstrates the importance of PNK in the response to genotoxic stress and the maintenance of genome integrity.

Tyrosyl-DNA Phosphodiesterase 1 (Tdp1)

DNA topoisomerase I forms DNA strand breaks during DNA relaxation and if these fail to religate then the topoisomerase becomes irreversibly attached to the 3'-DNA end. Yeast Tdp1 was originally isolated as an activity that hydrolyses the phosphodiester bond linking the tyrosine residue of topoisomerase to the DNA end that allows repair of the strand break.⁵⁰ The

gene for the human homologue (hTdp1) was subsequently cloned and was shown to encode a protein of 608 amino acids in length and approximately 68 kDa in size.¹¹ In addition to its tyrosyl-DNA phosphodiesterase activity, Tdp1 has a DNA double strand break specific 3'-phosphoglycolate activity and it is believed that Tdp1 is the only enzyme capable of processing this lesion on protruding 3'-termini of double strand breaks.^{11,51} However, Tdp1 differs in the mechanism employed by APE1 in that the enzyme generates a 3'-phosphate terminus that is a substrate for PNK, rather than the conventional 3'-hydroxyl end generated by APE1. The importance of Tdp1 in the processing of 3'-phosphoglycolate ends has recently been described.⁵² In this study, extracts from patients with spinocerebellar ataxia with axonal neuropathy (SCAN1) that have a mutation in the active site of Tdp1 and are thus inefficiently able to repair topoisomerase I-associated DNA damage,⁵³ were completely deficient in 3'-phosphoglycolate processing. However, the cells were only slightly radiosensitive, indicating an alternative, perhaps minor, repair pathway for DNA double strand breaks containing 3'-phosphoglycolate exists. It is possible that APE1 may account for this apparent lack of radiosensitivity since it was shown to be able to remove 3'-phosphoglycolate from double strand break-containing substrates, although a direct comparison of the kinetic parameters of Tdp1 and APE1 has not yet been done, so it is difficult to compare these activities.⁵² Interestingly, the yeast Rad1-Rad10 nuclease (ERCC1-XPF complex in human cells) that is essential for nucleotide excision repair can also cleave DNA containing 3'-phosphoglycolate ends.⁵⁴ Therefore, although a role for Tdp1 in the processing of 3'-phosphoglycolate containing double strand breaks in SCAN1 pathology has been proposed, most probably Tdp1 has its major role as a tyrosyl-DNA phosphodiesterase, releasing topoisomerase I irreversibly attached to the 3'-DNA end, as suggested in a recent study demonstrating that cells mutated in Tdp1 accumulate DNA SSBs.⁵⁵

Aprataxin

Another spinocerebellar ataxia syndrome, similar to SCAN1 and designated ataxia ocular apraxia (AOA1), has been described with mutations in the aprataxin gene. This gene is a member of the HIT domain superfamily of nucleotide hydrolases/transferases.⁵⁶ In addition to its central HIT domain, the aprataxin gene also contains a predicted zinc-finger at the C-terminus and an FHA domain at the N-terminus, that has significant homology with the FHA domain of PNK.⁵⁷ The human aprataxin protein is 342 amino acids in length and approximately 40 kDa in size. Furthermore, aprataxin was found to interact with XRCC1 although the relevance of this interaction during SSB repair remains to be elucidated.^{58,59} However, aprataxin has been demonstrated to be a novel protein that protects against genotoxic stress through protein-protein interactions with XRCC1, PARP-1 and p53.⁶⁰ Aprataxin is therefore a good candidate as an end processing enzyme, since it interacts with proteins involved in DNA repair and cells deficient in aprataxin are deficient in DNA SSB repair.^{60,61} However, a related biochemical activity of aprataxin is yet to be established.

Polymorphisms of Proteins Involved in the Processing of 3'-End Modified DNA Strand Breaks

It is thought that single nucleotide polymorphisms (SNPs) of BER genes may alter protein function and subsequently an individual's ability to repair damaged DNA, which in turn may result in increased genetic instability that could promote the progression of cancer. Various SNPs of proteins involved in the repair of oxidative damage have been widely investigated, especially with respect to cancer risk.⁶² A lot of work has focussed on SNPs of XRCC1 which is a key protein in DNA SSB repair.¹⁸ The most common SNPs for XRCC1 are R194W, R280H and R399Q and currently over 100 research articles worldwide have described links between these polymorphisms and various cancer risks.⁶³ For example, the R399Q polymorphism has been associated with an increased risk of breast,⁶⁴ lung,⁶⁵ and head and neck⁶⁶ cancer, whilst the R194W and the R280H polymorphisms have been associated with an increased risk in oesophageal squamous cell carcinoma,⁶⁷ and lung cancer,⁶⁸ respectively.

With respect to SNPs of proteins directly involved in the processing of 3'-end modified DNA strand breaks induced by oxidative damage, very little information is available. However, APE1 polymorphisms in the gene coding region have been detected and in particular the D148E polymorphism has been linked with increased risk of a number of cancers, including breast cancer.⁶⁹ The D148E polymorphism has been associated with an increased risk of lung cancer in the Chinese population, which is further increased when combined with the XRCC1 R399Q polymorphism.⁷⁰ Interestingly, five SNPs of the PNK coding region have been detected,^{71,72} but there is no current indication for a link between these and cancer.

A number of these studies appear contradictory with respect to whether SNPs are associated with an increased or decreased risk of cancer. Furthermore, larger and more comprehensive studies will be required to clarify the relationship between SNPs of proteins involved in the processing of 3'-end modified DNA SSBs and the links with cancer. These relationships can further increase in complexity when considering combinations of SNPs and should also take into account environmental risk factors, such as gender, ethnicity and lifestyle.

Conclusions

Reactive oxygen species generated as a consequence of endogenous oxidative metabolism or via exogenous factors, such as ionising radiation, can produce a wide variety of lesions in DNA. These include base damage, sites of base loss (abasic sites) and single and double strand breaks. However, reactive oxygen species can also form SSBs containing modified 3'-ends, such as those containing phosphate, phosphoglycolate and oxidative base damage and the majority of these small lesions in DNA are repaired by proteins involved in the BER pathway. It is apparent that due to the differing complexity of these lesions, they may require a subset of proteins for repair and indeed APE1, PNK and Tdp1 have been identified as 3'-end processors. APE1 has been characterised as the major activity in human cells involved in the repair of 3'-phosphoglycolate and also in the repair of 3'-8-oxoguanine residues in DNA SSBs that are resistant to DNA glycosylase excision, although it is predicted that APE1 may be able to recognise several oxidatively damaged bases on the 3'-end, thus being a nonspecific end processor. PNK has been characterised as the major 3'-phosphatase activity, although APE1 also has a weak activity for 3'-phosphate lesions and finally Tdp1 can excise 3'-phosphoglycolate lesions on double strand break ends.

Therefore, the formation of 3'-end lesions in DNA in human cells is apparent and repair systems have evolved the ability to cope with these damages through different repair proteins and mechanisms. The importance of these repair systems is demonstrated by the fact that a deletion in Tdp1 is associated with the neurological disorder SCAN1 and that a deletion in aprataxin, which is a good candidate for a 3'-end processor, is also associated with the neurological disorder AOA1. Furthermore, downregulation of APE1 and PNK has proved important in the sensitivity of cells to radiation and DNA damaging agents and indicates the vital role of these proteins in cell survival and genome stability. It remains to be seen whether other 3'-end processors will be identified and to establish the mechanism and roles of these proteins in DNA repair and the maintenance of genome integrity.

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CHAPTER 7

Oxidative Damage and Promoter Function

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Abstract

Evidence is accumulating that base damage, particularly that produced by oxidation reactions, can modulate DNA protein interactions and affect promoter function. Such lesions have the capacity to interfere with normal gene regulation through direct interactions with promoter elements, or indirectly by establishing new transcription factor (TF) binding sites. The direct “cis” effects are the most studied and offer the best evidence for oxidative damage interference in promoter function in vitro and in vivo. These studies reveal diverse responses of TF to oxidative damage in promoters that can have either no effect, induce a full or partial inhibition or, in some cases, actually enhance binding depending on the particular TF-promoter system under investigation and the location of the damage within the promoter element. Other, more hypothetical pathways are presented including the de novo production of new consensus binding motifs by oxidative damage/mutations and changes in promoter structure or sequence such that they acquire higher affinity for inappropriate transcription factors. The possibility of molecular hijacking is also discussed.

Introduction

High-affinity, sequence-specific DNA binding proteins that recognize multiple related sequence elements determine the precision of regulated events in eukaryotic cells. Transcriptional regulation is controlled by the activity of two types of trans-acting factors; one binding 5' to the transcription start site (promoter) and the other acting from some distance away (enhancer). The isolation and purification of eukaryotic transcription factors, such as Sp1, have made it possible to define multiple sites of DNA-protein interactions controlling gene activation. DNA damage within the coding region of a gene can inhibit its expression directly, by blocking the progression of RNA polymerase.¹ DNA damage can also alter protein-DNA interactions that may affect the regulation of gene transcription. These changes may affect gene expression through modifications in TF binding and contribute to genetic instability leading to cell transformation. Biological effects of damaged DNA in promoters may arise from modified transcription rates of active genes and/or erroneous activation of inactive genes. The non-random distribution of DNA damage in promoters suggests that a significant portion of the biological effects of genotoxic exposure, such as oxidative stress, may arise from defective gene regulation.

The notion that DNA damage-directed mutagenesis is the only important process in the initiation of carcinogenesis has been challenged by studies on the frequencies of the mutagenic and transformation processes.²⁻⁶ Several in vitro and in vivo studies show that the frequencies of transformation induced by a carcinogen are at least 100-fold higher than mutation frequencies

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induced by the same treatment.⁷⁻⁹ Thus, epigenetic mechanisms such as altered gene expression^{3,5} have been proposed to play a role in carcinogenesis. Dramatic changes in gene expression are clearly observed in several tumorigenesis models. At first glance, the long latency between carcinogen exposure and the appearance of malignant cells appears to argue against epigenetic mechanisms in carcinogenesis. However, several studies support the idea that transient, carcinogen-induced alterations in gene expression can become permanent.¹⁰

A small change in the expression of one gene can have a significant impact on cellular development. This is particularly important in a class of genetic loci known as selector genes, genes that act at the start of a developmental pathway and are, hence, necessary for development of an entire cell lineage or tissue. For example, expression of the *eyeless* gene, necessary for compound eye formation in *Drosophila melanogaster*¹¹ in selected larval tissues that normally give rise to other structures (e.g., wing, leg, and antenna) induces the formation of clearly defined ectopic eye structures in these areas. Thus, the inappropriate expression of a single gene leads to the replacement of one tissue with another. In another example, studies of "determination" in imaginal disc cells indicate that at a low frequency, cells of one imaginal type (e.g., antenna), when transferred to a metamorphosing larva, produce cells of another type (e.g., wing).¹²

The chief characteristic of such control circuits with bistable states is positive feedback or autoregulation. Many developmentally important genes, such as selector genes, have been described that are autoregulatory. For example, expression of cyclin E enhances expression from the promoter of the *E2F1* gene, and *E2F1* expression enhances transcription from the cyclin E promoter.^{13,14} It has been suggested that an autoregulatory circuit exhibiting bistable behavior can be switched from one stable state to another by a transient change in the concentration of a gene product. If the gene product also controls the transcription of another gene with pleiotropic effects (e.g., selector genes) stable inherited genetic alterations in cellular phenotype can result.

DNA damage affects TF-promoter function to varying degrees depending on the type of damage, its location in or around the promoter and the specific promoter targeted by the damage. Cis effects of DNA damage are those in which the damage is located within the promoter element and directly interferes with or modulates the interaction between the TF and its consensus binding sequence; trans effects involve an action spatially removed from the primary TF binding site. There are several ways in which DNA damage can effect changes in gene expression: First, the damage can either reduce or enhance TF binding to its consensus binding sequence; second, the binding of a TF to the altered DNA structure associated with a particular type of DNA damage may result in the production of new binding sites that effectively "hijack" the TF;¹⁵ third, DNA damage in a promoter element may render that region susceptible to binding by a TF not usually associated with that binding sequence resulting in the production of new binding sites affecting genes not normally regulated by that factor. Here, we will discuss evidence for these phenomena as they pertain to oxidative damage and speculate on the impacts these effects may have on human disease.

Nonrandom Induction of Oxidative Damage in Promoter Regions

Promoter regions that control eukaryotic genes are quite often either GC-rich or proximal to GC-rich tracks of DNA. Since guanine is particularly sensitive to oxidative damage, due to its extremely low oxidation potential, it is likely that guanine-rich promoter regions, such as the Sp-1 consensus sequence (5'-GGGGCGGGG-3') for example, would be particularly sensitive to, and selectively targeted by, oxidative stress. Using H₂O₂ and Cu(II), Oikawa and Kawanishi¹⁶ showed that DNA damage formed preferentially at the 5' site of 5'-GGG-3' in telomere sequences and that, specifically, 8-hydroxy-7,8-dihydroguanine (8-oxoG) formed more efficiently in telomere compared to nontelomere sequences.

In addition to sequence driven induction preferences, other factors such as DNA repair and TF binding itself can affect the site of damage formation (see review by Evans and Cooke).¹⁷

Work from Gerd Pfeifer's lab using ligation-mediated PCR (LMPCR) has shown that not only does DNA sequence influence sites of damage formation in certain genes (e.g., p53) but also that sequence-directed excision repair results in the accumulation and persistence of DNA damage at specific base locations.¹⁸ Although this has not been shown as yet for base damage, it is reasonable to believe that the structural and dynamic processes that determine site-specific accumulation of premutagenic bulky adducts may also be at work for oxidation products in promoters.

DNA structure and DNA-protein interactions can also influence the targeting of DNA damage. Additional work using LMPCR showed that UV photoproduct hotspots *in vivo* are associated with TF binding to promoters.¹⁹ Specifically, (6-4) photoproduct formation was enhanced up to 30-fold in the CC dipyrimidine site of the CCAAT box upstream from TF-bound PGK1, JUN and PCNA genes.²⁰ Work from our lab²¹ showed a diverse response for photoproduct induction in p50-bound NF- κ B gene. Whereas some of the sites within the 22-mer target DNA were suppressed by bound TF, some remained unaffected and one site showed increased damage formation. Although no data are currently available for the site-specificity of oxidative damage, it is likely that DNA-protein interactions will have significant influence over the extent and location of damage induction.

Cis Effects of Oxidative Damage on Promoter Function

Regulatory regions, such as gene promoters, are repaired by base and nucleotide excision repair and tend to accumulate DNA damage at specific sequence sites. Since eukaryotic transcriptional regulation is a complex process that is mediated by regulatory elements including cis-acting elements, trans-acting factors, trans-acting elements and transcription factor binding motifs, DNA damage in any of these regions has the capacity to disrupt this highly regulated process. Although it can be argued that functional DNA repair mechanisms remove damaged bases, evidence suggests that the efficiency and specificity with which cells repair such regions may affect transcriptional regulation. Of the different regulatory regions, cis-acting elements and trans-acting factors have been the most commonly studied. During transcriptional activation, gene-specific transcription factors bind very selectively to certain DNA sequences. However if this sequence is modified or disrupted in eukaryotes either by the presence of damaged or mutated bases (the latter arising from delay or failure to correctly repair) then DNA-protein interactions may become deregulated.

Several groups, including our own, have shown that damaged DNA bases disrupt DNA-protein interactions and therefore possess a potential to deregulate normal gene expression patterns.²²⁻²⁶ While in most cases DNA damage in the promoter region inhibits protein-DNA interaction, in some cases TF binding is enhanced and in yet other cases it appears to have no effect at all on the ability of TF proteins to bind DNA sequences. Studies on the effects of DNA damage on TF binding have utilized the common oxidative lesion 8-oxoG and the disruption of the sequence-specific binding of transcription factors AP-1, NF- κ B and Sp1. Consensus binding sequences for TF, such as Sp1, AP-1, and NF- κ B are GC-rich and, hence, vulnerable to common types of oxidative damage (e.g., 8-oxoG) (Table 1).

Work from our lab²³ and that of Jean Cadet²² showed that a single 8-oxoG modification within the Sp1 consensus-binding site inhibited the binding of Sp1 protein and that the degree of inhibition depended on the site of damage. It was evident from these studies that oxidation at bases located at protein-DNA contact points between Sp1 and its binding site are particularly disruptive to stable interactions. The observed difference in the ability of the same type of damage to affect DNA-protein interactions suggests that damage location with respect to DNA conformation may be important in the context of DNA-protein interaction. For example, the core of the GC box and the second and third zinc-finger domains appear to be particularly important for stable binding of Sp1.²²

Cis effects of AP-1 binding have also been examined and are similar to those observed for Sp1. We found that a single 8-oxoG at the unique G position within the AP-1 binding site was

Table 1. DNA sequences of the consensus binding sites of three transcription factors

Consensus Sequence	Transcription Factor	Protein
5' -CGC <u>TAC ATG ACT CAC</u> GCG CGA C-3'	AP-1	c-jun
3' -GCG ATG <u>TAC TGA GTG</u> CGC GCT G-5'		
5' -TGT GCA <u>GGG GAC TTT CCC</u> ACG C-3'	NF-κB	P50
3' -ACA CGT <u>CCC CTG AAA GGG</u> TGC G-5'		
5' -ATA CGT <u>ACG GGG CCG GGC</u> GTG C-5'	Sp-1	Sp1
3' -TAT GCA TGC <u>CCC GCC CCG</u> CAC G-5'		

The TF binding site is underlined.

sufficient to block TF binding.²³ These results were confirmed by Parsian and coworkers²⁶ who showed that a single 8-oxoG could partially inhibit AP-1 binding when located within or adjacent to the consensus binding sequence. They further showed that disruption of TF binding by an abasic site also displayed a position effect as well as a more overall inhibitory effect; 8-oxoadenine had no effect on AP-1 binding. Because of the importance of the thymine methyl group in transcription factor binding, Rogstad and coworkers²⁵ examined how substitutions in the AP-1 binding sites with 5-hydroxymethyluracil (oxidation of the methyl group of thymine) or uracil (misincorporation of dUMP into DNA) would affect the AP-1 transcription factor. Their results showed that both substitutions inhibited c-jun binding.

Studies on the effects of oxidative damage on p50 binding to the NF-κB consensus sequence are somewhat contradictory. The earlier of the two studies indicated that substitution of 8-oxoG for the third G residue in the G-run in the NF-κB binding site had no effect on p50 binding.²³ A later study, in which 8-oxoG was substituted for each of the four G residues, yielded a spectrum of responses with significantly increased binding observed when the first G residue was substituted, significantly reduced binding for the third G residue and no effect when 8-oxoG was substituted for the second and fourth G residues in this run.²⁷ Interestingly, base excision repair enzymes, such as Fapy glycosylase and 8-oxo-guanine DNA glycosylase, were prevented from repairing the lesion, consistent with the extent of bound p50 protein at the modified site. Although the differences between these studies are somewhat problematic, it should be noted that we observed significant differences between recombinant protein batches and the length of time proteins and buffers were stored frozen.

A unique class of oxidative DNA damage, namely 8,5'-cyclo-2'-deoxyadenosine was also tested for its ability to affect binding of TATA-binding protein to the TATA box. A single site-specific isomer of cyclo-deoxyadenosine reduced gene expression by about 75%.²⁴

Another cis effect of oxidative damage on transcriptional deregulation involves the bypass of small lesions by DNA polymerases resulting in enhanced mutagenicity at those sites. The effects of mutations on promoter function have not been examined to the extent that oxidative damage has. In one study, the pro-mutagenic mispair U:G actually increased c-jun binding to the AP-1 binding domain and inhibited activity of the repair enzyme, uracil DNA glycosylase.²⁵ Using another approach, the effects of polymorphisms on seven functional Sp1 TF binding sites was examined.²⁸ Three common polymorphisms in the MSH6 mismatch repair gene promoter region were identified and each functioned to inactivate a different Sp1 binding site. One polymorphic allele associated with a five-Sp1-site promoter was found in 16% of Caucasians and displayed 50% less promoter activity and was more sensitive to inactivation by methylation compared to the more common seven-Sp1-site promoter region. This polymorphic region resulted in reduced MSH6 expression at both the mRNA and protein levels. In another study, two naturally occurring point mutations in the transcription factor binding sites on the human retinoblastoma gene have been shown to decrease expression of the Rb gene product.²⁹

Although most of the studies on TF-promoter interactions have been performed using *in vitro* systems employing synthetic oligonucleotides and purified recombinant proteins, some evidence has been generated that validates these processes *in vivo*. Oxidative stress, including increased production of reactive oxygen species and reduced antioxidant defense mechanisms, has been implicated in the development of diabetic complications. TF binding to the Sp1 and NF- κ B promoters was examined in the livers and kidneys of diabetic rats using electrophoretic mobility shift assays and correlated with the level of 8-oxodG in these organs measured by HPLC-EC.³⁰ A significant decrease in the affinity of Sp1 to DNA was observed in DNA extracted from the kidneys of diabetic rats compared to the control group, correlating with higher levels of 8-oxodG measured in these same tissues. In another study, transcriptional profiling of the human frontal cortex was used to examine the relationship between aging, DNA damage and gene expression.³¹ They found that DNA damage is markedly increased in promoters of genes from the cortex of older patients and that the damage levels correlated with reduced expression of these genes. In addition, they found that these promoters were selectively damaged by oxidative stress in cultured human neurons with reduced base excision repair capacity.

DNA damage in gene promoters has the potential to affect disease states through deregulation of gene expression. For example DNA damage in the TF binding site can decrease or completely inhibit DNA-protein interactions and subsequent gene expression. Complete or incomplete silencing of genes that are involved in essential functions such as tumor suppression can be pivotal events in carcinogenesis. On the other hand, DNA damage in gene promoters can also create binding sites by modifying DNA conformation thereby turning on the expression of genes that would normally be quiescent. Such activation of oncogenes could also significantly affect the carcinogenic process.

Trans Effects of Oxidative Damage on Promoter Function

In contrast to what we are calling “*cis*” effects, “*trans*” effects involve an action spatially removed from the primary TF binding site (see Fig. 1). Hence, TF binding outside the promoter (III), binding to a promoter not normally associated with a particular TF (IV) or binding of a TF factor not normally associated with a particular promoter (V) would be considered “*trans*” effects of DNA damage on promoter activity. One mechanism of how such an effect might alter gene expression in the absence of permanent genetic change is called “molecular hijacking”.¹⁵ For example, heterologous DNA sequences modified by benzo[a]pyrene-diol-epoxide bind the Sp1 TF³² and cisplatin adducts in DNA bind the high mobility group protein, HMG1,³³ the human upstream binding factor³⁴ and the TFIIB/TBP basal TF.³⁵ In other words, DNA damage can produce new binding sites that effectively “hijack” transcription factors, resulting in loss of binding to the normal binding site (III) or substitution for a promoter region upstream from a gene not normally regulated by that factor (IV). In the cellular environment this could translate into the appropriation of a particular gene product at a time when it is required to regulate an essential gene function. Hijacking can also theoretically occur when damage within a heterologous promoter (not normally recognized by a particular TF) causes that promoter to be bound by a TF not normally associated with that promoter. In other words, the damaged promoter mimics another promoter. The potential consequences of such a scenario are complex: (1) the TF erroneously modulates a gene it does not normally regulate; (2) that TF is prohibited from regulating the gene it normally serves; and (3) the TF originally associated with the heterologous promoter is prevented from binding to that promoter and is free to associate with other DNA binding sites it may also be associated with.

We performed competitive binding assays to determine if 8-oxoG was capable of “hijacking” the transcription factors for AP-1, Sp1, and NF κ B with negative results.²³ These data suggest that recognition and binding of TFs may be predicated on the helical distortion in the promoter-binding site and may actually bind DNA damaged regions with helical structures that mimic promoter elements. It is possible that monobasic oxidative damage sites with minor effects on helical structure do not produce similar alternative binding sites for TFs. However,

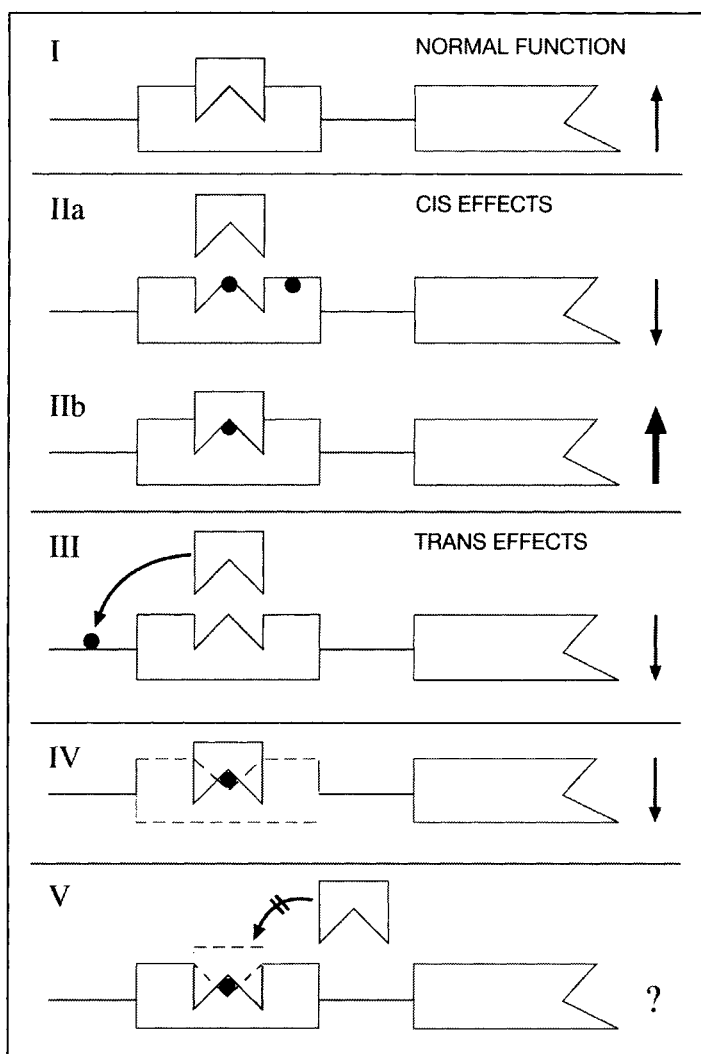


Figure 1. DNA damage effects on promoter function. Transcription factor (TF) binds undamaged promoter and (normally) increases activity (I); DNA damage in consensus binding sequence or proximal to binding site may either down-regulate (IIa) or up-regulate (IIb) gene transcription; Nonspecific binding of TF to DNA damage outside the promoter region (III) or within another promoter element (IV) may result in molecular hijacking and reduced transcriptional regulation of the correct coding sequence; DNA damage in the consensus binding sequence may appropriate a different TF resulting in potentially compromised regulation of more than one gene (V). Reprinted with copyright permission from The University of Texas MD Anderson Cancer Center.

there is some precedent that multifunctional proteins, such as the human ribosomal protein S3, have a high affinity for 8-oxoG in DNA and, indeed, may function in the base excision repair process.³⁶ In addition, it has also been shown that E2F1 binds BPDE-damaged DNA³⁷ and that in vivo expression of this TF in knockout and transgenic mice correlates with the efficiency of nucleotide excision repair. These data suggest an intriguing possibility that some TFs function not only in transcriptional regulation, but also in DNA damage recognition and

the early phases of DNA repair. Other work has indicated that, rather than enhancing DNA repair, TF binding to promoters containing DNA damage may shield the oxidized guanines from base excision repair proteins.²⁷ The role of TFs in DNA repair is complex and certainly warrants further investigation.

Conclusions

In conclusion, it is evident that DNA damage, particularly bulky adducts that significantly modify DNA helical structure, can interfere with normal gene regulation through interactions with promoter elements or with regions of damaged DNA that mimic TF binding sites. There is compelling evidence that smaller lesions, such as oxidation products and, particularly, the model lesion 8-oxodG, may have similar effects. This damage can either directly modulate the DNA protein interactions (cis effects) or it can modulate promoter-controlled gene regulation at some distance (trans effects). Cis effects are the best-studied pathway and offer a good argument for the significant role of oxidative damage interference in promoter function *in vitro* and *in vivo*. Although the mechanism(s) of cis effects are not well defined, they most likely involve structural changes in and around the promoter consensus sequence, especially at protein-DNA contact points. The responses of TF to oxidative damage in promoters are diverse and can have either no effect, can induce a full or partial inhibition or, in some cases, can actually enhance binding. These varied responses are dependent on various factors, including the particular TF-promoter system under investigation (i.e., Sp-1, AP-1, or NF- κ B) and the precise site of damage within the consensus binding sequence.

A perhaps more intriguing aspect of DNA damage consequences on gene regulation involves what we have termed "trans" effects. Several mechanisms are shown in Figure 1 (III-V). The scenarios shown in IV and V both involve modification of a binding site by oxidative damage. One reasonable mechanism for a trans effect is the *de novo* creation of a new consensus binding motif by either the damage itself (IV in Fig. 1) or by a mutation resulting from that damage (e.g., a T to G transversion). In this case, the TF is sequestered by an inappropriate binding site resulting in dysregulation of two genes; that is, upregulation of the gene controlled by the *de novo* binding site and reduced availability of the TF for binding to the original "correct" promoter element. Another reasonable scenario is shown in Figure 1 (V) where the original consensus sequence is altered in response to oxidative damage (or mutation) such that it is no longer recognized by the appropriate TF but rather binds another TF. This could result in pleiotropic effects similar to those discussed for IV, including misappropriation of a heterologous TF that may block the correct TF to one degree or another. A more hypothetical trans effect is molecular hijacking, where a TF interacts with DNA damage independent of the DNA sequence (i.e., TF binding site). This phenomenon has been well described for bulky DNA damage (e.g., BPDE and cisplatin adducts) but has not been detected for oxidative damage. Compared to most other disciplines, there is a relative paucity of data regarding oxidative damage and promoter function. The subject is compelling however, and it is hoped that some of the ideas set forth in this chapter will encourage future efforts in this direction.

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CHAPTER 8

Oxidative DNA Damage and Telomere Shortening

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Abstract

Telomeres are highly complex DNA-protein structures that protect the ends of chromosomes. A variety of DNA damage response and repair proteins are bound to telomeres and fulfil functions in the maintenance of the telomeric cap, in the protection of chromosomes from end-to-end fusion and, if telomeres become uncapped, in the induction and translation of a senescence signal. Telomeres shorten with cell division. This shortening is to a large extent caused by the accumulation of telomeric single-strand breaks. The telomeric DNA structure, with its high density of guanine repeats, results in an enhanced vulnerability towards DNA damage, especially oxidative damage. Moreover, repair of single-strand breaks in telomeres is less efficient than in the bulk of the genome, and this is dependent on TRF2 expression. Thus, telomeres act as cellular sentinels: by shortening and arresting cell proliferation in response to accumulated oxidative stress they protect cells and tissues from the adverse consequences of genomic damage.

Telomere Structure

Telomeres consist of repetitive DNA sequences, about 5000 to 15000 base pairs long in humans.¹ At the end of chromosomes they provide a protective cap for the gene-bearing DNA that lies further inside. Their sequence was first identified in *Tetrahymena* as “TTGGGG”. In both humans and the majority of mammals, the sequence is the similar “TTAGGG”. Telomeres show a distinctive G-C asymmetry with the G-rich strand running towards the 3' end and terminating in a single-stranded overhang. This DNA is supported by adjacent telomere specific proteins which together form the structure that we call the telomere.

In somatic human cells, telomeres shorten with every cell division (see below). In stem and germ cells short telomeres are replenished by the enzyme telomerase in late S phase. This enzyme is essentially a reverse transcriptase (hTERT subunit) that uses its RNA template (hTR subunit) to add “TTAGGG” sequences to the end of the telomeres.²

Whilst the proximal parts of the telomeres are organized in a conventional nucleosome structure, the end forms a so called “telomere loop” or “T-loop” (Fig. 1). The essential feature of this structure is a single-stranded overhang of the G-rich 3'-strand that is about 100 – 600 bp long in human cells. This single-stranded overhang can invade the telomeric double strand, probably by forming a DNA-triplet (also referred to as the “displacement loop” or “D-loop”),

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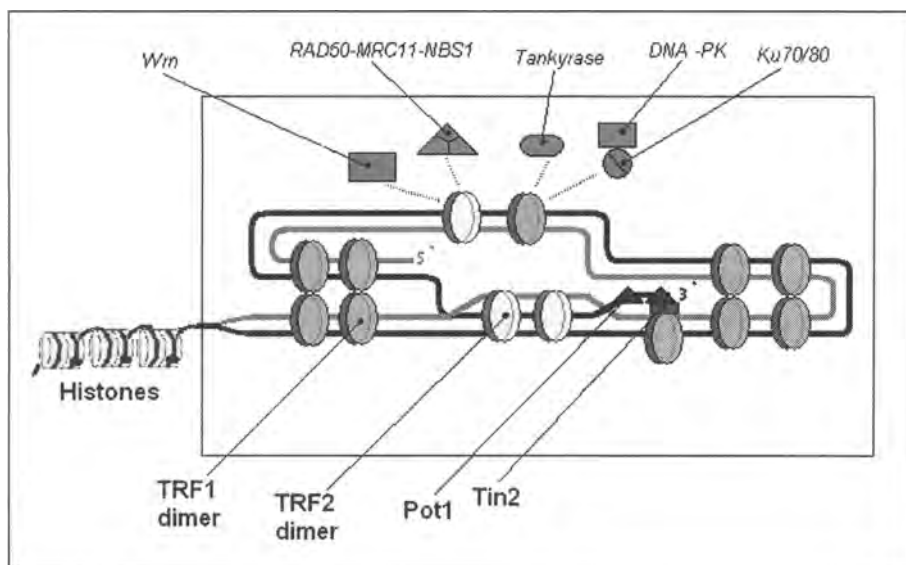


Figure 1. Telomere structure. The end of each chromosome is folded into a T-loop, supported by adjacent telomere binding proteins. Secondary telomere binding proteins (denoted in *italics*) are associated mainly through their interactions with the primary binding proteins TRF1 and TRF2.

which is stabilized by TRF2. This structure prevents the end from being recognised as damaged, unpaired DNA.

In addition to DNA processing enzymes like telomerase or those involved in general chromatin structure formation like histones, there are three further proteins interacting directly with the telomeric DNA: TRF2, TRF1 and Pot1. TRF2 is essential for forming and maintaining the T-loop structure. Its loss leads to telomere uncapping, an effect similar to that induced by short telomeres, i.e., induction of a DNA damage response and/or telomeric end-to-end fusions. Sequence specific binding to the double-stranded telomeric DNA by TRF2 dimers is mediated by a C-terminal Myb domain.³ These structures are similar to those of TRF1 but slight differences in DNA recognition and affinity can explain their different roles in forming the telomere structure.⁴

TRF1 is responsible for telomere length regulation in telomerase expressing cells and shows a molecular structure similar to TRF2. Although it binds only to double-stranded telomeres, it acts by attracting Pot1 to the single-stranded overhang when the concentration of TRF1 increases on longer telomeres. This leads finally to the blocking of telomerase. It might also help to stabilize the T-loop as shown in Figure 1.

With 100 to 600 bases, the G-rich overhang is exceptionally long in human telomeres as compared to some protozoa, where it is only 1/10 of this length.⁵ With such a size, single stranded DNA with a high G content that occurs in sequences of at least three consecutive guanine repetitions, is able to form higher order structures like those mainly known from RNA. This includes an asymmetric propeller shape formed in the presence of potassium ions,⁶ or DNA quadruplexes with either parallel or antiparallel orientations.⁷ These structures might stabilize the telomeric ends especially during opening of the T-loop, e.g., during S phase. They might also be important for intermolecular interactions between telomeres. In yeast, the overhang binding protein CDC13 has shown high affinity binding to quadruplex DNA. It is still unclear how far the quadruplex propeller fold supports or disrupts Pot1 binding. Ligands stabilizing the quadruplex structure have been shown to block telomerase access to the telomeres and are now in discussion as possible anti-cancer treatments.⁸

Other proteins form higher order telomeric protein complexes by interacting with the proteins directly bound to the DNA, such as certain DNA repair proteins, or Tin2. Tin2 forms the link between TRF1 and Pot1. A newly found protein (TINT1) might serve as an additional bridge between Pot1 and TRF2 and could help to further stabilize the closed loop structure.

Telomeres and DNA Repair

Telomeres are associated with a number of DNA repair enzymes that participate in either of the two main double strand repair pathways in higher eukaryotes,⁹ homologous recombination (HR) or nonhomologous end joining (NHEJ). These include WRN, BLM, Ku and DNA-PKc and the Mre11/Rad50/Nbs1 complex.

WRN and BLM belong to the RecQ family of DNA helicases, which are involved in recombinational repair of stalled replication forks and DNA breaks. Loss-of-function mutations in these genes lead to rapid loss of telomeric repeats, telomere dysfunction and chromosomal fusions.¹⁰ It appears that both the helicase and the exonuclease activity of the WRN protein are important in the controlled opening of T-loops for DNA replication. WRN shows high affinity to triple DNA structures like D-loops independent of their sequence.¹¹

Ku (Ku86/Ku70 heterodimer) functions together with DNA-PKc and the XRCC4/ligase4 complex in NHEJ repair of double strand breaks (DSBs).¹² The first step of NHEJ is the binding of Ku to a DSB, this way bridging the gap. It then attracts DNA-PKc, which initiates end processing for ligation by the XRCC4/ligase4 complex. The kinase function of DNA-PKc seems to play a crucial role in the organisation of this process. Although not all interactions are clarified yet, it was found that DNA-PKc is necessary for removal and relocation of key components.¹³ Its importance in repair is evident in DNA-PKc deficient organisms which display severe immunodeficiency and increased sensitivity to ionizing radiation.¹⁴

At telomeres, the complex is thought to be involved in prevention of telomeric end-to-end fusion.¹⁵ At the same time, NHEJ is also the main mechanism for DNA ligase IV-dependent chromosomal fusions that occur between uncapped telomeres.¹⁶ This suggests a selective regulatory switch, from preventing recombination to promoting it, together with the change from a functional/capped telomere to an uncapped one. In fact, Ku and DNA-PKc telomeric functions extend beyond mere prevention of chromosome fusion. They contribute actively to telomere capping and telomere length maintenance as well. While Ku acts as a negative regulator of telomerase, DNA PKc cooperates with telomerase in telomere elongation,¹⁷ so that nonfunctional DNA-PKc provokes telomere shortening and an earlier onset of age related pathologies.¹⁷ Although ligase IV is essential for the DNA repair function in the complex, its deletion has no comparable effect on the telomere associated function.¹⁸

The MR complex (Mre11/Rad50/Nbs1) acts in a chromosome-wide manner in recombination repair of DNA double-strand breaks.¹⁹ It is involved in the signalling that leads to the recruitment of repair enzymes, the induction of end-joining and repair, and cell cycle blockage. At uncapped telomeres, it has a similar function. Such dysfunctional telomeres activate signalling kinases like ATM or ATR, these phosphorylate histone variant H2A.X and recruit the MR complex into senescence associated DNA damage foci that signal irreversible growth arrest.²⁰ In functional telomeres, however, end-joining and fusions are inhibited rather than promoted by the MR complex and cell cycle arrest is not induced.²¹ Here, the MR complex seems to be associated with telomere maintenance and recruitment of telomere specific proteins, since mutations in MR proteins lead to shortened and fused telomeres.²² Clearly, there are parallels to the function of Ku and its selective regulation at functional/capped telomeres.

Telomere Shortening and DNA Damage

Telomeres in telomerase-negative cells shorten with cell division by relatively constant amounts, if measured by averaging over all telomeres, in a large number of asynchronously dividing cells, under constant stress and DNA damage levels. More recent data indicate a basal level of telomere loss per cell division, overlaid with occasional, random losses of large amounts of telomeric DNA.²³ The first explanation as to why telomeres would lose bases constantly was

found in a mechanism known as the “end-replication effect”.²⁴ While DNA-polymerase can act continuously on the leading DNA strand to the very end, the gap left by the RNA primer most distal on the lagging strand cannot be filled in and results in continuous shortening of the newly synthesized 5'-strand. The lost fragment is at least as large as the RNA primer (8 – 12 nucleotides), but can be larger, if the RNA primer was not placed at the very end.²⁵

In addition to the end replication problem, there is an elaborate processing of the telomere end after replication, even in telomerase-negative cells, serving to maintain single-stranded G-rich overhangs at all telomeres.²⁶ It is not clear how this processing impacts upon telomere loss, as telomere shortening and overhang length are not correlated.²⁷

Finally, it became clear that telomere shortening is highly sensitive to endogenous oxidative stress. Oxidative damage has a major contribution to average telomere loss in most cells, even under standard (culture at 37°C in 20% oxygen, 5% CO₂) conditions^{28,29} and might explain the occurrence of individual ‘ultra-short’ telomeres.²³ The role of oxidative damage in telomere shortening will now be discussed in more detail.

Early observations showed different rates of cell growth under different oxygen conditions. Cells grown under mild chronic hyperoxia (40% oxygen) displayed inhibited proliferation³⁰ and achieved lower numbers of total cumulative population doublings. The same effect was observed after repeated treatments with oxidants like tert-butylhydroperoxide³¹ or hydrogen peroxide in low concentrations.³² In contrast, reduction of oxidative stress by culture under low oxygen, or treatment with antioxidants, prolonged the replicative lifespan in primary cell cultures.³³

Additional investigations showed that cells grown under conditions of relatively high oxidative stress lose telomeres by up to one order of magnitude faster than comparable cells under low oxygen conditions.²⁹ This suggested oxidative damage as a major factor in telomere maintenance which could be verified by investigations of the influence of mitochondrial ROS production on telomeres (Passos et al submitted for publication, 2005). However, telomere loss was only found in replicating cells, indicating that the connection between oxidative damage and telomere shortening was not via direct induction of double-strand breaks (which would lead to sequence loss even without replication) but rather via damage to single strands of DNA²⁸ which happens to be the type of damage that occurs under mild oxidative stress conditions.

Further insight was provided by measurements of the frequency of single-strand breaks (SSB) and telomere shortening rates in growing fibroblasts, at the end of a prolonged growth arrest and after resumption of cell division. Whilst cells in growth arrest showed a high frequency of telomeric SSB and no telomere shortening, the situation was reversed shortly after the first round of DNA replication. The frequency of telomeric SSB decreased to basal levels, but the rate of telomere shortening was significantly increased and would reverse to normal levels only later. The conclusion is that oxidatively induced telomeric SSB cause a significant part of the telomere shortening observed under standard cell culture conditions.³⁴ This, and the accelerated telomere shortening under increased oxidative stress or the significant slow-down by treatment with antioxidants, free radical scavengers and overexpression of antioxidant enzymes,³⁵⁻³⁷ suggests a mechanism by which single strand breaks promote telomere shortening due to an early replication abort when the polymerase encounters a break close to the end of the telomere,³⁸ as shown in Figure 2A.

Telomeres Are Vulnerable to Oxidative Stress-Induced Single-Strand Breaks

Stretches of DNA containing consecutive guanines or thymines are highly sensitive to various forms of insult including UV crosslinking (thymine dimerisation),³⁹ chemical crosslinking by e.g., cisplatin⁴⁰ or alkylation.⁴¹ However, the oxidative conversion of guanine into 8-oxo-7, 8-dihydroguanine (8-OH-Gua) is probably the most important lesion in telomeres.⁴² This type of damage is preferentially generated in regions with repeats of consecutive guanines. A triplet of guanines has a much higher reactivity in duplex DNA, compared to single guanines. In fact, telomeres are more sensitive towards oxidative stress than regions in the overall genome.⁴¹ For instance, when oxidative stress is induced by treatment with hydrogen peroxide,

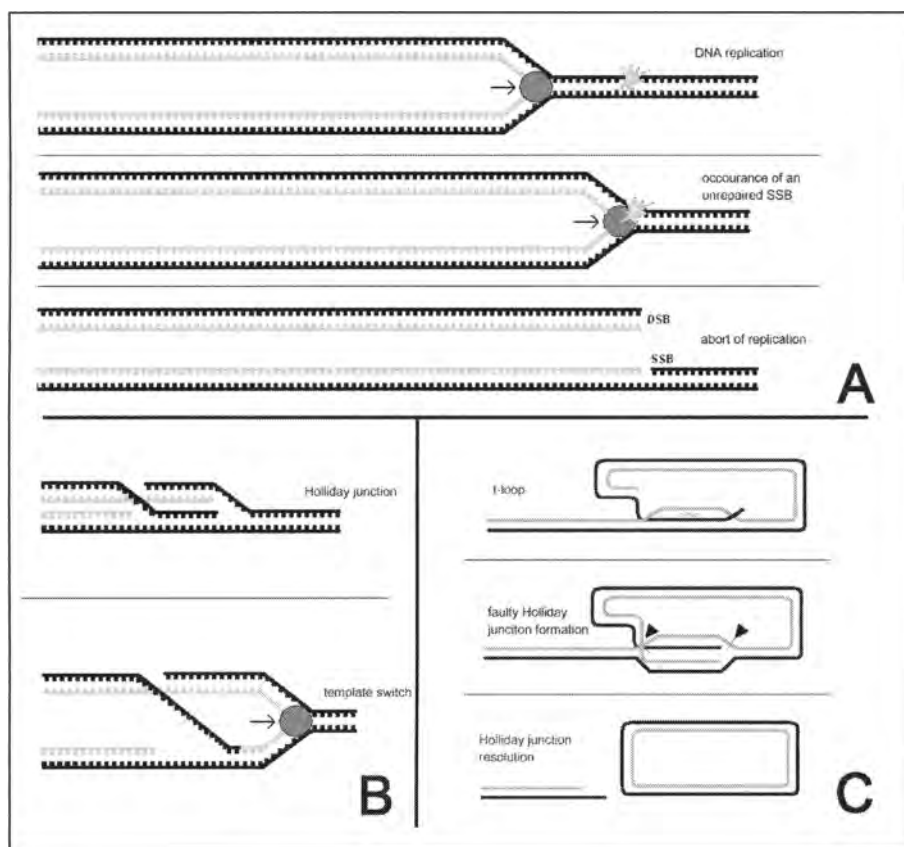


Figure 2. Effect of single strand breaks during DNA replication. A) A single strand break (SSB) in the leading strand that is encountered during DNA replication may result in replication abort when the replication associated SSB-repair failed. The outcome is loss of the remaining sequence on one strand, while on the other strand the SSB between the freshly synthesized strand and the old strand remains. (grey: newly synthesized strands, black: parental strands). B) The replication fork can be reestablished through the formation of a Holliday junction. This prevents loss of terminal DNA. This process leads to a switch in the template. C) Holliday junctions can also form within the T-loop if branch migration into the D-loop occurs. Resolution of the Holliday junction requires two strand incision events (triangles) and results in telomere truncation and the formation of circular telomeric DNA (grey: 5' end, black: 3' end; after).⁵³

in combination with Cu(II), predominant formation of 8-OH-Gua at the 5' site of the 5'-GGG-3' in the telomeres is observed.⁴²

The presence of 8-OH Gua might have multiple effects on telomere structure and function; for example by affecting the formation of intramolecular G quadruplexes in the telomeric overhang,⁴³ which might compromise the ability to form T-loops and thus contribute to accelerated telomere loss. If 8-OH-Gua formation occurs in the 5' position, which supports quadruplex formation, it inhibits telomerase activity significantly.⁴⁵ Moreover, the binding of TRF1 and TRF2 is also affected by 8-OH-Gua formation. Binding assays have shown that single 8-OH-Gua lesions introduced in a defined telomeric substrate reduced the percentage of bound TRF1 and TRF2 by at least 50% and multiple 8-OH-Gua lesions had an even more dramatic effect.⁴⁴ This would again be expected to destabilize T-loops.

8-OH-Gua lesions are repaired by base excision repair (BER), the first step of which is the conversion of the lesion into a single-strand break by 8-oxoG-DNA glycosylase (OGG1) and APE1. This repair step appears to be unhampered in telomeres. In cells that are photosensitized by riboflavin, UV treatment results both in five-fold increased 8-OH-Gua formation and OGG1 activity on telomeric sequences, as compared to nontelomeric DNA.⁴⁵ This finding confirms the enhanced vulnerability of telomeres to 8-OH-Gua lesions and the fact that the conversion of 8-OH-Gua lesions into single-strand breaks under different oxidative insults is at least as fast and as frequent as in the bulk of genomic DNA.^{34,41} Interestingly, further repair of SSB in telomeres, in growth-arrested cells, seems incomplete. In many human fibroblast strains, telomeres show about 10-fold higher frequencies of SSB under basal conditions (growth under 20% ambient oxygen), as compared to interstitial repetitive DNA sequences.^{34,41,46} After an oxidative challenge, some of the formed telomeric SSB remain unrepaired as long as the cells are held in growth arrest, even over long observation periods in stark contrast to the rest of the genome.⁴¹ Even under basal cell culture conditions, the frequency of telomeric SSB increases with time in growth arrested cells.³⁴ These observations suggest two conclusions: First, there is a telomerespecific deficiency in the late steps of BER when it comes to SSB repair; second, accumulation of telomeric SSB in G0 or G1 might contribute to telomere shortening during DNA replication.

Telomere-Specific Deficiency of SSB Repair

If accumulation of SSB in telomeres (due to BER or by direct damage to the sugar-phosphate backbone of the DNA) contributes significantly to telomere shortening, the question arises why telomeric SSB are less well repaired than elsewhere in the genome.

Genome-wide SSB repair is fast and highly efficient.⁴⁷ Its structural details are well characterized at the molecular level. Briefly, poly(ADP-ribose) polymerase 1 (PARP-1) is activated by auto-ribosylation at SSB and adds poly-ADP-ribose to the damaged region, which acts as a signal and opens the chromatin structure by applying negative charges. Activated PARP-1 interacts with the scaffold protein XRCC1 that binds the strand opposite of the break. It remains there during the repair process and acts as a platform for all subsequent repair enzymes, orchestrating all necessary actions. The termini of SSB are often damaged and show 5'-hydroxyl and/or 3'-phosphate ends. 3'-phosphate ends are unable to serve as primers for polymerase and need therefore to be processed. 3'-phosphate groups are removed by endonucleases APE1/HAP1 or the mammalian T4-PNK homolog which has also 5'-kinase activity to process 5'-hydroxyl ends. After this step DNA polymerase-beta (Pol-β) docks onto the XRCC1/DNA platform and fills the gap by introducing a new base. Un-processed 5'-hydroxyl ends can still be removed by the AP-lyase activity of Pol-β. The last step is the ligation by Lig3α which is also stabilized by XRCC1. Finally, XRCC1 dissociates from the site. It has been proposed that the poly(ADP-ribose) serves as a source of ATP for the ligation step.⁴⁷ This targeted repair mechanism is replication-independent and accounts for up to 80% of genomic SSB repair. The remaining SSBs in the genome are repaired in a replication-dependent fashion using PCNA, Pol-Δ/ε, Fen1 and Lig1.

Current data indicate that SSB repair is compromised at telomeres,⁴¹ although the specifically compromised step is not clear yet. Furthermore, the precise effects upon telomeres, of overexpression or deletion of any of the genes involved in SSB repair have not been reported so far.

Knockout of PARP-1 influenced telomere length in one mouse model, but not in another strain.⁴⁸ Moreover, the reported effect seemed to influence a telomere length set-point in the embryo or the zygote rather than telomere shortening, suggesting that PARP-1 knockout had no major effect on telomeric SSB repair.

Tankyrase (TANK) 1 and 2 are PARPs specific to the telomeres. TRF1 is a target for TANK1. Poly ADP-ribosylation of TRF1 by TANK1 removes it from the DNA. This involves TANK1 in the regulation of telomere length in telomerase-positive cells.⁴⁹ However, there are no data so far to suggest that tankyrases can play a role in telomeric SSB repair, or maintenance of telomere length in telomerase-negative cells.

The single most important protein known to influence telomere shortening in telomerase-negative cells is TRF2. Fibroblasts overexpressing wild-type TRF2 shorten their telomeres faster than control cells.⁵⁰ At the same time, TRF2-overexpressing fibroblasts show decreased rates of SSB repair in telomeres, but not in the total genome. This telomeric repair deficiency was specific to TRF2, as it was not found in cells overexpressing either hTERT, the catalytic subunit of human telomerase, or TIN2, a TRF1-interacting telomeric protein (Richter et al manuscript in preparation). TRF2 is the major protein conferring stability to the T-loop. It is conceivable that a highly condensed T-loop structure is hardly permissible for the significant conformational changes resulting from poly-ADP-ribosylation and binding of the scaffold protein XRCC1. Another possibility comes from the fact that Pol- β can interact with TRF2.⁵¹ So far, this interaction has been demonstrated to occur at nontelomeric sites in Pol- β -overexpressing cells, resulting in dysfunction of TRF2.⁵¹ Whether TRF2/Pol- β interaction can also functionally compromise Pol- β , and whether Pol- β is in fact functionally retarded at telomeres, has not yet been examined.

Possible Mechanisms of Stress-Dependent Telomere Shortening

How can the presence of a SSB in the telomere lead to telomere shortening? Sites of DNA damage can stall the replication fork. In the worst case scenario, this might lead to replication abort, which would result in one shortened daughter telomere and the replication of the break in the other (Fig. 2A). Even a temporary inhibition of progression of the replication fork, along the leading strand, might have serious consequences in the vicinity of the end of the DNA template, because synthesis would still continue along the opposite parental strand. A polymerase that has reached the very end of one template strand might not be able to resume translation synthesis on the opposite strand.³⁸

A telomeric SSB that is not bypassed appropriately by the replication complex (and thus leads to a replication abort as shown in Fig. 2A), can easily form a Holliday junction (Fig. 2B) initiating HR (Homologous Recombination). This way replication can be re-established, although the resolution of the Holliday junction by HR involves a DNA template switch.⁵² Given that the frequency of SSB at telomeres is about 10-fold higher than elsewhere in the genome, we suggest that this type of recombination-related HR might occur with high frequency at telomeres, thus explaining the need for functional recombination complexes to maintain telomere length and integrity.

Interestingly, HR can also contribute to telomere shortening. The telomeric D-loop, in which the G-rich single-stranded overhang is tucked back into the double strand (Fig. 1), shows similarities to a Holliday junction. As recently suggested, simple strand migration might turn the D-loop into a Holliday junction.⁵³ The resolution of the Holliday junction requires first cleavage of both C-rich strands at the crossing-over point, which is presumed to be performed by XRCC3, a component of the Holliday junction resolvase. Complete truncation of the telomere and the separate occurrence of a circular telomeric DNA of the size of the T-loop additionally requires nicking of the D-loop⁵³ (see Fig. 2C).

If the Holliday junction is not immediately resolved, it can be used as a mechanism for telomere extension by rolling circle replication (see Figure 2C for Holliday junction formation in telomeres and Figure 2B for replication after Holliday junction formation. Notice that at telomeres the two strands are connected by a loop).⁵⁴ This mechanism might have relevance for telomere maintenance in ALT (Alternative Lengthening of Telomeres) cells.

A mutant form of TRF2 (TRF2^{ΔB}) fosters sudden telomere loss and T-loop-sized circle formation, suggesting that the deleted section of TRF2 inhibits faulty Holliday junction formation on telomeric D-loops. However, sudden deletions of telomeres approximating to the size of a T-loop,²³ as well as T-loop-sized circular telomeric DNA,⁵³ have been found at low frequency in normal human cells harbouring intact TRF2. Thus, homologous recombination at the D-loop might be important as a mechanism causing sudden telomere deletions in normal cells.

A telomeric SSB might provide a preferential entry point for strand invasion (Fig. 2B). It is also possible that a SSB in the D-loop might contribute to strand migration and cleavage of

telomeric circles through HR. The probability that the D-loop encompasses a SSB increases with oxidative stress. Thus, we suggest that oxidative stress-dependent telomeric SSB accumulation contributes to sudden telomere loss.

Conclusion

Considering all of the evidence together, telomeres seem to act as sentinels for DNA damage.²⁹ Due to their SSB repair deficiency, they are the first to be affected by oxidative stress, resulting in accelerated telomere loss and finally uncapping, which triggers cellular senescence. This limits the amount of DNA damage that can be fixed as mutation during DNA replication elsewhere in the genome, including coding regions, and thus limits the risk of cancer in a multicellular organism.

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CHAPTER 9

Oxidative Damage and Repair in the Mitochondrial Genome

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Abstract

DNA is constantly exposed to damaging agents from both endogenous and exogenous sources. If this damage is not repaired, it can lead to mutations and result in cellular dysfunction, including uncontrolled cell proliferation. Thus, in order to maintain the integrity of the genome, a complex network of DNA repair pathways exists to remove the majority of deleterious lesions. However, DNA repair may occasionally fail or become limited due to an excess DNA damage, resulting in DNA damage accumulation.

Characterization of DNA repair mechanisms has generally focused on these processes in nuclear DNA. While the interest and research efforts concerning DNA repair in mitochondria have increased, much is still to be learned. In this chapter, we will focus on current knowledge of oxidative DNA damage and repair systems in the mitochondrion, make comparisons to what is known about repair in nucleus, as well as discuss the link between dysfunctional mitochondria and human disease.

Introduction

Normal cellular metabolism is well known as a source of endogenous reactive oxygen species (ROS) and it is these, usually nonpathogenic, cellular processes that account for the background levels of oxidative DNA damage detected in normal tissue. Pathways and events that produce ROS include mitochondrial and peroxisomal metabolism, enzymatic synthesis of nitric oxide (NO), therapeutic drugs, oxidizing agents, and ionizing radiation. The reaction of ROS with pyrimidines and purines produces a variety of DNA lesions,¹ with 8-oxoguanine (8-OH Gua) by far the most studied, but not necessarily the most important, DNA lesion. If cells did not have cellular defenses, such as low molecular weight antioxidants, enzymatic antioxidants, and DNA repair, levels of such oxidatively modified bases would quickly represent the majority of bases in DNA.

Superoxide radicals ($O_2^{\cdot-}$) are normally eliminated by superoxide dismutase (SOD), which generates the less reactive hydrogen peroxide (H_2O_2) and O_2 . The H_2O_2 is further converted to H_2O and O_2 by catalase. SOD activities are present in both mitochondria (SOD2, Mn-SOD), cytoplasm (SOD1, CuZn-SOD), and extracellularly (SOD3, EC-SOD).² A large number of other factors also contribute to cellular defense against ROS, for example dietary antioxidants, thiols, polyphenols, enzyme-bound minerals, and antioxidant enzymes. All of these are important for the protection of amino acids, proteins, and lipids.³ However, none of these cellular systems repair DNA damage and thereby prevent permanent genetic alterations.

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Mitochondrial respiration is the major source of endogenous ROS, including $O_2^{\bullet-}$, H_2O_2 , and hydroxyl radical ($HO^{\bullet}$). Under normal physiological conditions, electrons leak from the electron transport chain converting about 1–2% of oxygen molecules into $O_2^{\bullet-}$.^{4–7} Thus, increased mitochondrial metabolism generates higher than normal levels of ROS. Equally, inhibition of mitochondrial metabolism can also increase ROS production^{8–10} suggesting that correct mitochondrial function is important for mutation avoidance. The importance of ‘accurate’ mitochondrial function in preventing mitochondrial-mediated mutations is supported by several studies showing that mitochondrial dysfunction is promutagenic and multiple pathways are involved in this phenotype.^{11–13} However, mitochondria are not only involved in the generation of oxidative damage, they also have an effect on repair of DNA lesions. It was shown that a human cell line depleted of the mitochondrial genome showed impaired nuclear repair of H_2O_2 -induced DNA damage.¹⁴ Along these lines it was reported that preexposure of human cells to H_2O_2 suppresses DNA repair of alkylation damage,¹⁵ suggesting that extensive oxidative damage inhibits cellular repair systems. One possible target could be the mitochondrial DNA polymerase γ since it has been shown that this enzyme is a target for oxidative damage, which might result in reduced replication of the mitochondrial genome, as well as DNA repair capacities.¹⁶ Overall, these results indicate that correct mitochondrial function is important for both optimal nuclear and mitochondrial repair of oxidative DNA damage as well as for the prevention of excess oxidative damage.

Repair of Oxidatively-Damaged DNA in the Nucleus

Repair of damage in nuclear DNA is carried out through six major repair pathways: (1) direct reversal (DR), (2) nucleotide excision repair (NER), (3) base excision repair (BER), (4) mismatch repair (MMR), (5) recombinational repair (RER), and (6) translesion synthesis (TLS). Several of these repair systems contain secondary subpathways directed against specific types of DNA damage. However, all the pathways follow the same overall strategy: Localization and elimination of damage, followed by restoration of the DNA helix. All six pathways are found, with a certain variation, in both prokaryotes and eukaryotes suggesting that maintenance of genomic integrity is a fundamental process for any cell.¹⁷ Below we focus on the repair pathways that are known to play a role in repair/processing of oxidative damage.

Base Excision Repair (BER)

The BER pathway deals with those lesions that involve relatively subtle modifications to individual bases. As with most repair processes, the BER system is highly conserved amongst organisms, from bacteria to humans, and this repair system is believed to be the main pathway for repair of oxidatively damaged DNA.^{18–21} Traditionally, the BER process has been divided into two mechanistically different subpathways known as short- and long-patch repair, respectively. Common for both these repair processes is that they are initiated by a DNA lesion specific glycosylase, and differences between the two repair processes are the downstream reactions.²² The first step of BER is performed by a DNA glycosylase that recognizes and removes the abnormal base by hydrolysis of the N-glycosidic bond between the sugar-phosphate backbone and the base. This results in an abasic site (AP-site) that is recognized and cleaved by an AP endonuclease, which introduces a DNA strand break 5' to the baseless sugar. Alternatively, the AP-site is processed by an AP-lyase activity of the glycosylase creating a 3'-fragmented deoxyribose. Finally, a DNA polymerase fills the gap, and the nick is sealed by DNA ligase.^{21,17}

One characteristic of BER is the redundancy of DNA glycosylases in the initial step of this repair process. The initial enzyme in BER, a DNA glycosylase, is capable of recognizing a particular subset of base alterations. These DNA glycosylases are small monomeric proteins (20 to 40 kDa) that function without additional cofactors and access nucleotides via ring flipping.²³ DNA glycosylases fall into two categories, monofunctional and bifunctional depending on their catalytic properties. Monofunctional glycosylases cleave the glycosylic bond leaving an AP-site, while bifunctional glycosylases contain an additional AP-lyase activity

creating a 3'-fragmented deoxyribose.²³ DNA glycosylases can also be subdivided into 4 groups according to their main substrate: excision of uracil, uracil-containing mismatches, alkylated bases, and oxidized bases, the latter of which includes OGG1, MYH, NTG1, NTG2 and NEIL1.²³

Oxidizing species can give rise to a large number of DNA alterations that can be both cytotoxic and mutagenic. The best studied of these DNA oxidation products is 8-OHGua, which can mispair with adenine. The enzyme 8-oxo-guanine glycosylase 1 (OGG1) is a bifunctional glycosylase that removes 8-OHGua paired with cytosine and is also active against formamidopyrimidines that arise through ring fragmentation of purines.^{23,24} The monofunctional MutY homolog (MYH) glycosylase removes adenine that has been incorrectly incorporated opposite 8-OHGua. Cytotoxic oxidative lesions that would block transcription and replication are removed by the NTG1 (NTH) and NEIL1 glycosylases, which both have broad substrate specificities. NTG1/NTH mainly removes thymine glycol, but is also capable of removing ring-fragmented pyrimidines, 5-hydroxycytosine, 5-hydroxyuracil, dihydroxyuracil, dihydroxythymine and urea.²³ NEIL1 is a bifunctional glycosylase that acts as a backup for both OGG1 and NTH/NTG1 by removing thymine glycol, 5-hydroxyuracil and 8-OHGua paired with C, G or T.²³ The overlap in substrate specificity of the various BER glycosylases provides greater protection for the genome. Several of the DNA glycosylases that take part in repair of oxidized bases, including 8-OHGua participate in both short- and long-patch repair.^{25,26} Interestingly, an enzyme with MutY-like specificity has not been found in *Saccharomyces cerevisiae*. Instead, it was shown that the DNA mismatch repair (MMR) protein complex Msh2-Msh6 (MutS α) bound 8-OHG/A basepairs and that mutations in *MSH2* and *MSH6* in combination with mutations in *OGG1* caused a synergistic increase in G/C to T/A transversion mutations.²⁷ These results suggest that MMR can act as a functional homolog of MutY in *S. cerevisiae* and perhaps also in other organisms which lack MutY enzymes.

After DNA glycosylase activity, the resulting AP-site must be processed in order to generate a free 3'-OH terminus. AP-sites created by a monofunctional glycosylase are processed by the AP-endonuclease (APE1, APN1), which nicks 5' to the AP-site.²³ APE1 is also active at AP-sites formed by bifunctional glycosylases, where it utilizes a 3'-phosphodiesterase activity to remove the remaining deoxyribosephosphate moiety, leaving a free 3'-OH terminus.²³ In this case APE1 interacts directly with Pol β , which inserts a single nucleotide, and with XRCC1/LIGIII, which reseals the remaining nick.²³ This pathway is known as the short-patch BER pathway. APE1 incisions at AP-sites are repaired via both long- and short-patch repair. In short-patch repair Pol β also utilizes a 5' phosphodiesterase to clear the deoxyribosephosphate moiety and in long-patch repair Pol δ/ϵ aided by PCNA and RFC synthesize a 2 to 6 nucleotide track, thus creating an ssDNA overhang, which is subsequently cleared by the FEN1 endonuclease and the remaining nick sealed by LIG1.²³

Other enzymes involved in repair of 8-OHGua DNA lesions are MutT and its homologs in eukaryotic cells (MTH1 and hMTH1). The activity of MTH1 inhibits erroneous incorporation of 8-OHGua into DNA by degrading 8-OHdGTP to 8-OHdGMP. Similarly, MTH1 can also degrade 8-OHdATP to limit misincorporation of this modified nucleotide into DNA.²⁸

Interestingly, a DNA glycosylase-independent incision activity of oxidative DNA damage by Nfo/Apn1-like enzymes has been identified and provides an alternative pathway to traditional BER.^{29,30} This repair activity has been named nucleotide incision repair (NIR) and could provide an explanation for the DNA repair proficiency of DNA glycosylase-deficient mutants as a back-up repair pathway.

Translesion Synthesis (TLS)

The TLS repair pathway is yet another system that enables cells to repair lesions derived from DNA oxidation and other DNA lesions, that escape the vigilance of the generally efficient DNA repair systems.³¹⁻³³ Spontaneous mutation rates are decreased in cells deficient in TLS and, therefore, it can be speculated that spontaneous mutations in nuclear DNA of yeast

and mammalian cells are attributed to the activity of the TLS pathway.³⁴ TLS occurs when the replication machinery, upon encountering a lesion, has, or somehow acquires, the ability to copy the damaged template directly by incorporating a nucleotide opposite the modified base. TLS is potentially mutagenic because it often incorporates incorrect nucleotides and is described as an error-prone DNA repair pathway.³⁵ In *S. cerevisiae*, three proteins, Rev1, Rev3 and Rev7 constitute the major components of TLS. The *REV1* gene product possesses deoxycytidyl transferase activity whereas Rev3 and Rev7 proteins are the subunits of DNA polymerase ζ . The function of these proteins is conserved across the species.³¹ The yeast TLS polymerases Pol η and Pol ι are both able to insert a C opposite 8-OHGua and in Pol η Ogg1 deficient cells, there is a synergistic increase in spontaneous mutations.^{36,37} The human and mouse DNA polymerase κ (DINB, damage-inducible B) can support TLS across damage induced by DNA oxidation³⁸⁻⁴¹ and it has been shown that human Pol η (XPV) is not essential for the bypass reaction but when present, it is involved in bypass of 8-OHGua *in vivo*.⁴² Not all the TLS polymerases bypass 8-OHGua, some only extend from nucleotides inserted opposite this lesion suggesting a concerted action of the various TLS polymerases in processing oxidized DNA lesions.⁴³ The mechanism underlying the choice of polymerase is not yet clear.

Mismatch Repair (MMR)

The MMR pathway has been shown to play a role in mutation avoidance caused by DNA oxidation.^{27,44,45} Interestingly, Ni et al²⁷ showed that, when MMR-deficient yeast strains are grown anaerobically, the mutation frequencies are greatly reduced. The fact that MMR acts at sites of oxidatively damaged DNA suggests an interaction between BER and MMR, which is supported by results showing that there is a synergistic increase in mutation rates in *MLH1 OGG1*, *MSH2 OGG1* and *MSH6 OGG1* double mutant strains compared to the single mutants.^{44,46} One characteristic of MMR-deficiency is microsatellite instability (MSI), which can be caused by DNA oxidation.^{47,48} There are two obvious explanations for this (1) the MMR pathways repair oxidatively damaged DNA and/or (2) the MMR pathway is inactivated by oxidative processes, as for example the mitochondrial DNA polymerase γ , see above. In support of the latter, it was shown that low levels of H₂O₂ inactivate MMR activity, and that this is most likely due to oxidative damage to the MMR protein complexes hMutS α , hMutS β , and hMutL α .⁴⁹ However, another study has shown that cells treated with H₂O₂ show decreased, or no effect, on mutation frequencies at mononucleotide repeats; although a small increase in mutation frequency was observed at CA repeats.⁵⁰ Another study showed that, in human cells, H₂O₂ treatment caused less cytotoxicity in MMR-deficient cells than in those proficient in MMR, and that growth of MMR-defective cells in the presence of ascorbate reduced both the spontaneous mutation rate, as well as microsatellite instability. The induction of mutations by exogenously added H₂O₂ was significantly suppressed by antioxidant pretreatment, suggesting that oxidative damage contributes significantly to the spontaneous mutator phenotype in MMR-defective cells.⁵¹

The MMR system may act on mismatches involving oxidized bases, such as 8-OHGua/A and 8-OHGua/C mismatches.^{27,44,45,52,53} The hMSH2-hMSH6 complex hydrolyses ATP in the presence of 8-OHGua/A mismatch indicating that MMR processes this mismatch.⁴⁵ However, it is unclear if the oxidized bases are directly repaired by the MMR pathway, or if the lesions are recognized and marked for repair by other pathways. The fact that hMYH and hMSH6 physically interact⁵² suggests a direct interaction between BER and MMR at least under certain conditions (Fig. 1A,B). Since both are involved in recognition and/or repair of 8-OHGua/A it is tempting to speculate that these repair proteins play complementary roles in repair of 8-OHGua depending on when in the cell cycle the 8-OHGua/A mismatch occurs. If the human MMR system is analogous to that in bacteria, one would expect that it is only active during DNA replication, i.e., in S-phase. If an 8-OHGua/A mismatch is generated due to incorporation of 8-OHdGTP opposite adenine during DNA replication the 8-OHGua lesion would be present in the newly synthesized strand and, therefore, a substrate for removal by MMR. In this case a

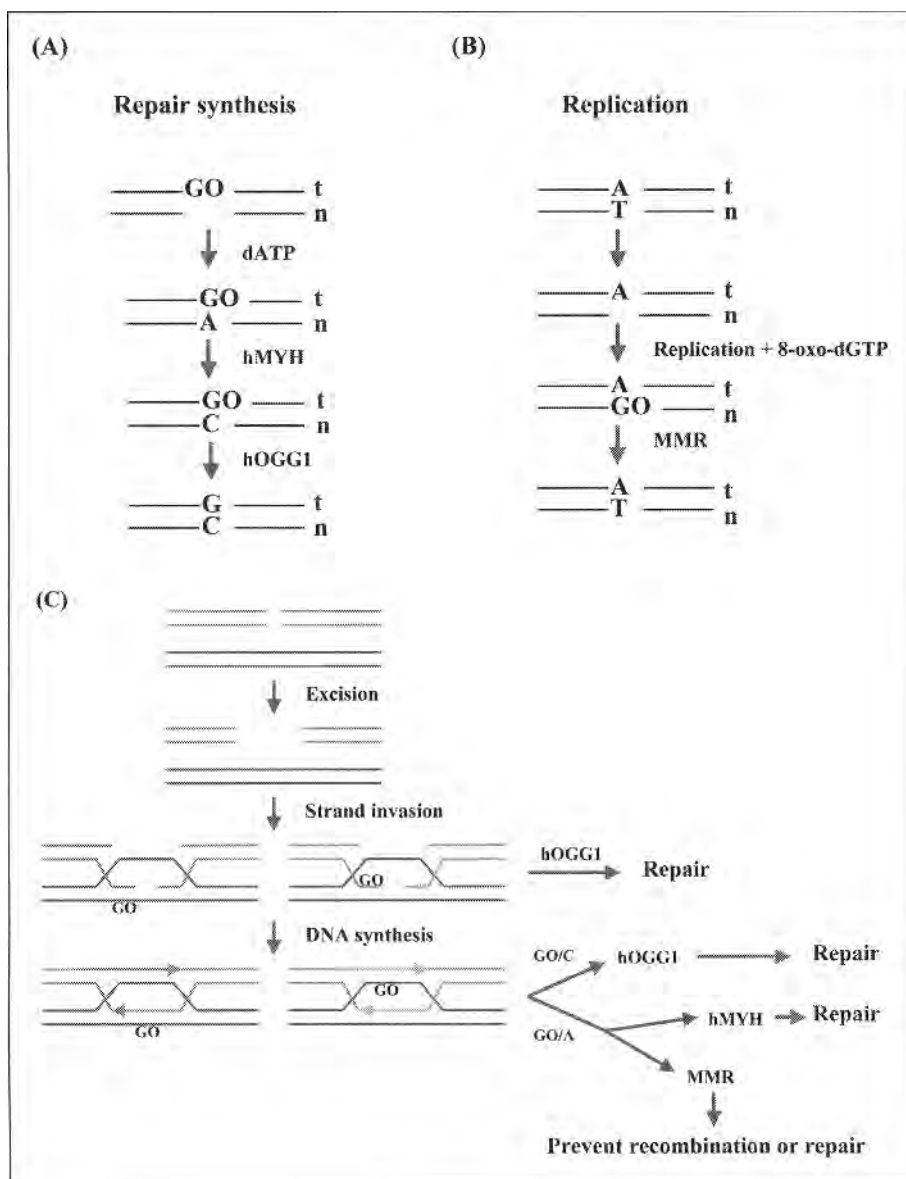


Figure 1. Mismatch repair processing of oxidative DNA damage. GO, 8-OHGua; t, template strand; n, nascent strand; MMR, mis-match repair. (Adapted from Rasmussen LJ. Oxidative damage to DNA and its repair. In: Singh KK, ed. *Oxidative Stress, Disease and Cancer*. Singapore: Imperial Press, ©2006, with permission from World Scientific Publishing Col. Pte. Ltd.)

thymidine would be inserted and the repair process completed. In this scenario there would be no need for hMYH to act on this lesion and, in support of this, it has been shown that 8-OHGua/A mispairs, where 8-OHGua is present in the parent strand, are not substrates for repair by the MMR system.⁵⁴ In contrast, when adenine is incorporated opposite an 8-OHGua DNA lesion

present in the template strand the adenine needs to be replaced by cytosine to avoid mutation. In this scenario there would be a need for hMYH to replace the adenine with a cytosine in the newly synthesized strand to avoid mutations.⁵⁵ The question is when would there be a need for hMYH and why is there a physical interaction between hMYH and hMSH6? One explanation is that the 8-OHGua/A mispairs that are substrates for hMYH are not formed during DNA replication but rather during repair synthesis or recombination.⁵⁶ During recombination MMR proteins play a major role in preventing recombination between substrates that contain numerous mismatches (homologous recombination) and, therefore, one could also speculate that MMR could block recombination between substrates containing abnormal bases like 8-OHGua (Fig. 1C). The importance of hMYH in this situation would be to initiate repair and generate a DNA template, which is error-free after recombination. The protein-protein interaction between hMYH and hMSH6 could serve either to make sure that the two proteins are physically close to each other and/or to regulate the enzymatic activity of one or both enzymes. On the other hand, if 8-OHGua/A is generated during repair synthesis MMR would not be active and hMYH would be the sole enzyme to initiate removal of the oxidized DNA lesion. Interestingly, MLH1 has been shown to interact with Ntg2.⁵⁷ The Ntg2 is a nuclear thymidine glycol DNA glycosylase that has a broad spectrum of DNA lesions as substrates.²⁶ The interaction between these repair proteins could be explained as above, for hMYH and hMSH6, suggesting a model where "repair factories" are present in the cell. Interestingly, the closely related Ntg1 glycosylase, which is present both in the nucleus and mitochondria, and the Ogg2 glycosylase as well as MLH1 have been found in mitochondria isolated from yeast.^{58,59} However, no physical interaction could be detected between these proteins using the two-hybrid assay.⁵⁷

Repair of Oxidatively-Modified DNA in Mitochondria

Base Excision Repair (BER)

In contrast to other repair pathways, BER is relatively well described in mitochondria.^{17,60-64} In human nDNA, 8-OHGua DNA lesion persistence is primarily prevented through BER by the OGG1, MYH, and MTH1 enzymes. Two isoforms of OGG1 exist, α OGG1 and β OGG1, which both arise from alternative splicing of the nuclear encoded transcript.⁶⁵ The β OGG1 contains a mitochondrial targeting sequence (MTS) in the NH₂-terminal part of the protein and can be identified in the mitochondrial matrix but this protein has no glycosylase activity (Klungland et al, this volume).⁶⁵ The importance of OGG1 in repair of mtDNA has been emphasized by several studies. One of these showed that mitochondria, isolated from OGG1-deficient mice, were defective in incision of 8-OHGua DNA lesions. In contrast the amount of 8-OHGua lesions in nDNA was unaffected by the absence of OGG1, implying the presence of alternative 8-OHGua repair mechanisms, which are specific for nDNA.⁶⁶ Similar results have been obtained in *S. cerevisiae*, where a strain lacking Ogg1 showed a two-fold increase in mtDNA deletions.⁵⁸ Furthermore, human cells defective in mitochondrial OGG1 activity showed decreased survival after oxidative damage, which could be reversed by introducing *OGG1* fused with a MTS.⁶⁷ Overall, these results suggest that OGG1 plays a major role in protecting the mitochondrial genome from oxidative stress, mutation, and preventing cell death.

8-OHGua is not the only substrate for OGG1. It has been shown that α OGG1 removes 8-OHAde, which is responsible for both A to G as well as A to C mutations.⁶⁸ Furthermore, whilst mitochondrial extracts isolated from mouse liver cells remove 8-OHAde from 8-OHAde/C mispairs, the same activity is not found in mitochondria isolated from the isogenic OGG1-deficient cells.⁶⁹ No incisions of double-stranded DNA substrates containing 8-OHAde/G mispairs were detected in either wildtype or OGG1-deficient mitochondria.⁶⁹ These results indicate that the mitochondria isolated from OGG1-deficient cells do not repair 8-OHAde/G mispairs in mtDNA but instead these DNA lesions could contribute to mutations in mtDNA.

In *E. coli* the MutY glycosylase removes adenine from 8-OHGua/A mispairs. This glycosylase activity has been conserved through evolution and is also found in mammalian cells, where it is known as MYH and is a monofunctional glycosylase.⁷⁰ Knockout studies in mice show that together with OGG1 MYH forms a cooperative defense against G:C to T:A transversions.⁷¹ Several splice variants of MYH have been identified in mammalian cells⁷² where some of the best characterized encode proteins of 52 kDa, 53 kDa, and 57 kDa in size. The 57 kDa form of MYH is found in mitochondria and localizes in the mitochondrial matrix.^{73,74} However, in mouse ES (embryonic stem) cells two isoforms of MYH have been identified in mitochondria.⁷⁵ In all studies the mitochondrial isoforms of MYH specifically remove adenine from both G/A and 8-OHGua/A containing oligonucleotides and do not contain an AP-lyase function.^{72,74,75} There exists some uncertainty about the actual size of the active mitochondrial form of MYH since another group found that a 38 kDa mitochondrial protein removes adenine from 8-OHGua/A mismatches and is recognized by both MYH and MutY antibodies.⁷⁴ However, a subsequent study showed that mitochondria isolated from rat brain cells contained a 57 kDa protein that was recognized by MYH specific antibodies.⁷⁶ Increased ROS production is accompanied by an increase in MYH levels in mitochondria^{76,77} suggesting that the level of MYH in mitochondria is specifically regulated in response to mtDNA damage.

Oxidative damage to mtDNA can lead to several forms of oxidized pyrimidines, for instance thymine glycol (Tg), which is only mildly mutagenic⁷⁸ but can block the progress of both DNA and RNA polymerases.⁷⁹ Accumulation of Tg in mtDNA can thus lead to disturbances in transcription as well as DNA synthesis, which could lead to mitochondrial DNA depletion. In many organisms, the EndoIII family of glycosylases removes Tg DNA lesions. In mammalian cells, the primary EndoIII homolog is named NTH1, a 36 kDa bifunctional glycosylase.⁸⁰ In addition to Tg, eukaryotic NTH1 recognizes and removes a number of other pyrimidine oxidation products, such as formamidopyrimidines, dihydroxyuracil, dihydroxycytosine, and urea.⁸¹ Human NTH1 has been predicted to contain a MTS.⁷³ However, contradicting results have been published concerning the subcellular localization of NTH1.^{73,81} In mammalian mitochondria an endonuclease activity that removes Tg and contains an AP-lyase function has been reported.⁸⁰ A subsequent study suggests that the mitochondrial Tg endonuclease is indeed a splice variant of NTH1.⁸² In yeast, the two NTH1 homologs *ngt1* and *ngt2* have been identified and it was shown that Ntg1 localizes to the nucleus as well as to mitochondria.⁸³ Further indication of NTH1 activity in mitochondria came from studies of OGG1-deficient mice liver cells, which showed residual activity against formamidopyrimidines in mitochondria.⁸⁴ However, the importance of NTH1 deficiency on mitochondrial repair remains unclear, although it has been reported that HeLa cells expressing *E. coli* EndoIII with a mitochondrial targeting sequence showed increased Tg removal from mtDNA, as well as increased cell survival after menadione treatment.⁸⁵ In addition, NTH1-deficient mice show no activity against either Tg/C or Tg/A mispairs in liver cell mitochondria.⁸²

After recognition of the DNA lesion by glycosylases, the next step in the BER pathway involves an AP-endonuclease (APE) to clear the 3'OH-terminus to accommodate polymerase activity. In *X. laevis* mitochondria almost all endonuclease activity on an AP-site is due to APE activity⁸⁶ and in rat thyroid gland cells it was shown that APE/Ref-1 localizes to mitochondria, despite the lack of an NH₂-terminal MTS.⁸⁷ A putative NH₂-terminal MTS sequence is present in the human APE2 gene and in HeLa cells GFP-tagged APE2 is located in mitochondria.⁸⁸ The level of mitochondrial APE/Ref-1 in human Raji cells is increased after treatment with H₂O₂.⁸⁹

The mitochondrial polymerase γ , encoded by the nuclear gene *MIP1*, is the sole active polymerase present in mitochondria and participates in all mtDNA metabolic processes.⁹⁰ Mice carrying a homozygous loss of function mutation in the exonuclease domain of Poly, display a higher rate of mtDNA base substitutions and deletions, which leads to the early onset of a wide range of age-related diseases.⁹¹ The mammalian Poly possesses AP-lyase activity, which allows it to remove 5'-deoxyribose phosphate (dRP) moieties created by APE or bifunctional

glycosylase activity.⁹² This allows Poly to function in the place of Pol β in the mitochondrial BER pathway. However the catalytic rate of dRP lyase by Poly is slower than that of Pol β , suggesting that only a low frequency of AP-sites can be processed in mtDNA compared to nDNA.⁹² Poly itself is a target for oxidation by ROS in the mitochondrial matrix. When human fibroblasts were treated with H₂O₂, Poly was shown to be one of the major oxidized proteins in the mitochondrial matrix both in vitro and in vivo.¹⁶ Oxidization leads to a decline in the catalytic activity of Poly, suggesting that high ROS levels may lead to reduced replication fidelity and thus interfere with mtDNA integrity and maintenance,¹⁶ which could lead to a vicious cycle of ROS generation.^{93,94}

The final step in the BER pathway is sealing of the nick left behind by Poly, which requires a mitochondrial DNA ligase activity. DNA sequence analysis of the human *LIG3* gene revealed an alternative downstream translation initiation site and a putative MTS and it was shown that this variant localized to mitochondria.⁹⁵ The importance of LIG3 in maintaining mtDNA stability was confirmed using antisense strategy to lower LIG3 levels, which showed a significant increase in nicks in mtDNA compared to the wildtype cells.⁹⁶ These experiments indicate that DNA ligase III functions as the central DNA ligase in mitochondria.

Sanitization of the dNTP Pools

To avoid the subsequent misincorporation of modified dNTPs into mtDNA, with potentially mutagenic effects, a number of triphosphatases sanitize the mitochondrial dNTP pool. In cytosol 8-OHdGTP is hydrolyzed to 8-OHdGMP by MTH1, in order to avoid the incorporation of 8-OHGua opposite A, which can cause C to A transversions. The MTH1 protein mainly localizes to the cytosol and to the mitochondria.⁹⁷ The MTH1 protein shows some activity against 8-OHdATP suggesting that MTH1 also participates in avoiding misincorporation of oxidized adenine.⁹⁸ Recent studies have revealed an additional MutT homologue MTH2 in mice, which is also capable of hydrolyzing 8-OHdGTP.⁹⁹ MTH2 was proposed to have a redundancy role, backing up MTH1 activity and explaining the lack of phenotype in MTH1-deficient mice.⁹⁹ However it is still unclear if MTH2 is present in mitochondria. Furthermore, the mitochondrial MTH1 is induced by ROS¹⁰⁰ indicating that mitochondrial splice variants of nuclear DNA repair genes can be specifically regulated in response to mitochondrial ROS levels.

Recombinational Repair (RER)

The repair of double-strand DNA breaks (DSB) by homologous recombination is essential for the maintenance of genome stability.¹⁰¹ The DSBs may arise as a consequence of replication fork collapse at sites of oxidative damage and increased levels of DSBs that may induce hyper-recombination, leading to deleterious genetic changes.^{56,102-104}

One protein involved in mitochondrial recombination is the Pif DNA helicase, which exists in two forms generated through the alternative use of two AUG codons where the longer form localizes to mitochondria.¹⁰⁵ However, it still remains to be shown if this protein is involved in oxidative damage induced recombination. One more mitochondrial protein involved in recombination is Mhr1, that encodes a protein of unknown function. *S. cerevisiae* cells deficient in *MHR1* are defective in mitochondrial recombination and an active Mhr1 protein is required for mitochondrial function by reducing the level of spontaneous oxidative damage in mtDNA.¹⁰⁶ These results link recombination to repair of oxidative damage in mitochondria.

Mismatch Repair (MMR)

In eukaryotic MMR, a mismatch is first recognized and bound by either the hMSH2-hMSH6 (hMutS α , MutS homologs) or the hMSH2-hMSH3 (hMutS β , MutS homologs) complex. The hMLH1-hPMS2 (hMutL α , MutL homologs) complex is believed to create a contact between an endonuclease (MutH homolog) and the hMSH2-hMSH6/hMSH2-hMSH3 complexes. The endonuclease activity is thereby activated and a single nick is introduced into the newly

synthesized strand. The DNA double helix is unwound by helicases and exonucleases remove the bases on the newly synthesized strand. Finally, DNA polymerase fills in the excision tract and DNA ligase closes the nick.^{107,108}

To date, it is still unclear whether mammalian mitochondria harbor MMR activity. However, recent data⁵⁹ show that the proteasome of *S. cerevisiae* contains several components of the MMR pathway such as Msh1 (MutS homolog) and Mlh1 (MutL homolog). The Msh1 protein has previously been shown to localize to mitochondria and inactivation of the *MSH1* gene resulted in large scale mtDNA rearrangements suggesting that Msh1 is indeed involved in repair of mtDNA.¹⁰⁹ A homolog to yeast Msh1 has not been identified in humans suggesting that other proteins are responsible for this repair activity in humans. Interestingly, Chen et al¹¹⁰ have identified the MSH2 protein, a central player of the MMR system, in rat mitochondrial lysate. Further support for MMR activity in mitochondria came from Mason et al¹¹¹ who showed that purified mammalian mitochondria possess an activity that repairs mismatched substrates in vitro. The MMR pathway has been shown to play a role in mutation avoidance caused by oxidative damage.^{27,44,45} A recent study analyzed the functional overlap between MMR and Ogg1.¹¹² It was shown that adenine was misincorporated opposite to 8-OHGua in Ogg1-deficient *S. cerevisiae* cells resulting in 50-fold increase in transversion mutations in a mitochondrial marker gene.¹¹² Overexpression of wildtype Msh1, but not an inactive Msh1 mutant protein, reduced the amount of transversions in mtDNA suggesting that Msh1-mediated MMR acts on 8-OHGua DNA lesions in mitochondria.¹¹² Interestingly, overexpression of Msh1 increases cells with dysfunctional mitochondria measured as an increase in petite formation¹¹² suggesting a role for Msh1 in recombination of mtDNA.

Imbalanced Repair in Mitochondria

It has previously been shown that overexpression of hOGG1 in mitochondria protects cells against oxidative stress.^{67,113} In contrast, overexpression of other DNA glycosylases such as *N*-methylpurine glycosylase (MPG) in both nucleus and mitochondria increases sensitivity to alkylating agents.^{34,114} Our results also show that overexpression of MGMT in mitochondria increases protection of cells from the cytotoxic effect of the alkylating agent MNNG (Rasmussen and Rasmussen, 2006, submitted).

Mitochondria-Mediated Mutagenesis

Mitochondria are a major source of ROS production and dysfunction of this organelle is implicated in mitochondrial-mediated nuclear DNA mutagenesis.¹¹ Whether ROS generated during mitochondrial respiration is a major source of spontaneous mutations in nDNA is still debatable.¹¹⁵ It has been demonstrated that inactivation of the yeast *REV1*, *REV3* and *REV7* genes suppressed the ρ^0 -mediated mutator phenotype suggesting that ρ^0 cells generate DNA damage, which is converted into mutations by the TLS pathway. The *REV1*, *REV3* and *REV7* genes are conserved between yeast and humans and it is, therefore, tempting to speculate that the human *REV1*, *REV3* and *REV7* proteins may also be involved in mitochondria-mediated mutagenesis. While the TLS pathway generates mutations in cells with dysfunctional mitochondria, it does not generate mutations in antimycin A treated cells.¹¹ This drug is a specific inhibitor of the quinone reduction site; it binds to the *bc₁* complex, and blocks electron flow at complex III. These data suggest that nuclear DNA damage arising from mitochondrial dysfunction is complex and is converted into mutations by mechanistically different routes.

Mitochondrial Dysfunction and Human Disease

Inactivation of mouse *MYH* results in a minor 2-fold mutator phenotype.¹¹⁶ Nevertheless, it has been suggested that mutations in hMYH predispose to colorectal cancer, based on findings that missense mutations in this gene were identified in individuals with high occurrence of multiple adenomas and colorectal carcinoma.¹¹⁷⁻¹¹⁹ The missense mutation in the murine gene

mMYH G365D, corresponding to one of the human germline mutations G382D found in cancer patients, was shown to be defective in 8-OHGua/A but not 8-OHGua/C activity in vitro indicating MYH-deficiency but OGG1-proficiency.¹¹⁶ These results suggest that the human G382D missense mutation affects glycosylase activity and, therefore, these individuals are more sensitive to genetic changes caused by DNA oxidation. A role for MTH1 in cancer development is supported by experimental data using *MTH1* knockout cell lines as well as mice. The MTH1-deficient mice showed greater number of tumors in lung, liver, and stomach compared to wild-type mice.¹²⁰

Nitric oxide is a signaling and effector molecule that contributes to multiple physiological and pathophysiological processes in cells. Both NO and peroxynitrite are capable of inhibiting hOGG1 activity indicating that NO directly inhibits a key BER enzyme responsible for repair of 8-OHGua.¹²¹ NO-mediated inhibition of base excision DNA repair may promote the persistence of oxidatively damaged DNA and thereby contribute to mutagenesis.

It was shown that the reduced level of Frataxin protein, involved in the human disease Friedreich's ataxia, causes oxidative damage to mitochondrial proteins, induces mitochondrial dysfunction and nuclear DNA damage in a *RAD52* mutant, deficient in double-strand break repair. These results suggest that mitochondrial dysfunction generates damage to both mitochondrial and nuclear DNA and that these lesions are converted into DSB that are substrates for repair by the Rad52 protein.¹²²

Acknowledgements

This work is supported by NIH grants R01 CA113655 and R01 121904 awarded to K.K. Singh and a Danish Cancer Society grant awarded to L.J. Rasmussen.

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CHAPTER 10

The Role of Oxidative Damage to Nucleic Acids in the Pathogenesis of Neurological Disease

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Abstract

Oxidative stress involving reactive oxygen species (ROS) and reactive nitrogen species (RNS) is integral to the process of aging and age-related diseases such as Alzheimer disease (AD), Parkinson's disease, and amyotrophic lateral sclerosis (ALS). Oxidative stress-induced modification of nucleic acids impacts on the function of the cell, which can have an important role in the cause of AD. ROS induces hydroxylation of nucleic acid bases (e.g., formation of 8-hydroxy-2'-deoxyguanosine (8-OHdG) from deoxyguanosine), and RNS induces their hydroxylative deamination (e.g. cytosine to uracil conversion). 8-OHdG is commonly used as a marker of DNA damage in AD and other age-related diseases, and is approximately 10-fold higher than other oxidized bases. It is present in significant amounts in the mitochondrial and nuclear DNA of AD brains, as compared to control cases. Mitochondrial DNA is relatively more prone to damage as it is exposed to increased concentrations of ROS. In addition to transversion mutations of nucleic acid bases, oxidative stress-induced DNA damage results in deleterious DNA-DNA and DNA-protein crosslinking. DNA-DNA crosslinks may also be initiated by RNS-induced deamination of nucleic acid bases. ROS can also modify amyloid- β , through the oxidation of its constituent methionines to the corresponding radical cations, which initiate free radical chain reactions leading to its aggregation. Nucleic acids are also damaged through the mediation of advanced lipid peroxidation products, such as trans-4-hydroxynonenal (HNE) and 4-oxo-2-nonenal (ONE), which result in the formation of the corresponding "propano-" and "etheno-" adducts. The substantial DNA damage in AD reflects impaired mitochondrial function in the cases of AD, which results in increased ROS/RNS and decreased ATP formation, the latter impacting on the DNA repair.

Introduction

An imbalance between the production of ROS and cell antioxidant defenses results in oxidative stress, which is invariably involved in the process of aging and age-related diseases such as AD, Parkinson's disease, and ALS.¹ ROS and RNS, the source of oxidative stress, are produced

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during mitochondrial respiration,² as well as normal cellular and xenobiotic metabolism. Most of the initially recognized ROS are free radical species, based on which, the 'free-radical theory of aging' assumes that cellular damage accumulates as a result of free-radical induced reactions.³⁻⁵ Extra- and intracellular exposure to various noxious insults and metal-catalyzed reactions can lead to the generation of ROS. ROS are involved in the modification of cellular biomolecules, such as DNA, proteins, and lipids.^{6,7}

In addition to mutation, which is most commonly considered, oxidative modification of DNA can have other, broad-ranging effects upon the function of the cell, promoting microsatellite instability, DNA inhibiting methylation, and accelerating telomere shortening.^{8,9} The resulting oxidative DNA damage may have an important role in the pathogenesis of many diseases, including AD.¹ Especially in the case of AD, oxidative stress is regarded as one of the earliest pathological events,^{10,11} involving metabolic changes,¹² redox-active metals^{13,14} as well as other factors.¹⁵ Among other event, oxidative modifications of nuclear and mitochondrial DNA are thought to exacerbate AD.

RNS also cause DNA damage through hydrolytic deamination of the DNA bases (vide infra). Another route by which DNA may be modified is via reaction with advanced lipid peroxidation end products (ALEs) such as trans-4-hydroxy-2-nonenal (HNE), through the formation of DNA-HNE adducts (vide infra).¹⁶ Our recent review outlines the mechanisms and repair of the DNA damage involving ROS, RNS, and ALEs.¹⁷ In this overview, we will summarize recent developments on the effects of oxidative stress on DNA damage in AD and the cellular defense mechanisms for the attenuation of oxidative stress and maintenance of genomic stability.

Oxidative Stress and Free Radical Species

Reactive Oxygen Species

Reduction of molecular oxygen by reduced transition metal ions (e.g., Fe^{2+} or Cu^+) gives superoxide radical anion ($\text{O}_2^{\cdot-}$), which is protonated at pH below 6 to give the hydroperoxyl radical ($\text{HOO}^{\cdot}$). This, in turn, is converted to hydrogen peroxide (H_2O_2) by further metal ion catalyzed reduction, followed by protonation. Alternatively, superoxide dismutase (SOD) can also catalyze the transformation of superoxide radical anion into hydrogen peroxide. Nonenzymatic superoxide dismutation is very rapid and will occur in biological systems when the concentration of SOD is low. However, this can generate singlet oxygen, which, in itself, can damage DNA. The superoxide anion and hydrogen peroxide by themselves are not highly oxidizing species, but the Fe^{2+} or Cu^+ dependent Fenton reaction of H_2O_2 results in the formation of the highly reactive hydroxyl ($\cdot\text{OH}$) radical. The decomposition of peroxyntirite, a reaction product of nitric oxide (NO) and superoxide radical anion, also generates hydroxyl radicals.¹⁸ The generalized reaction sequence for the formation of hydroxyl radicals and H_2O_2 is shown in Figure 1.

ROS and in particular, hydroxyl radicals can oxidize methionine to give various oxidation products, such as methionine radical cation ($\text{MetS}^{\cdot+}$) or methionine sulfoxide. The former, generated by the one electron oxidation involving hydroxyl radicals, may initiate free radical chain reactions, and may have relevance in the oxidative stress associated with amyloid- β in which methionine is a constituent amino acid.¹⁹

Reactive Nitrogen Species

RNS, especially peroxyntirite, can initiate DNA damage. Peroxyntirite is derived from the reaction of nitric oxide (a free radical) with the superoxide radical anion. Rapid protonation of peroxyntirite anion *in vivo* gives peroxyntirous acid (ONOOH), which acts as an electrophilic nitrating agent for tyrosine and tryptophan side-chains in proteins. Decomposition of peroxyntirous acid can generate hydroxyl radicals, which can subsequently damage DNA (vide supra). Alternatively, nitric oxide can also react with hydroxyl radical to give nitrous acid (HNO_2)²⁰ which can cause diazotization of the primary amino groups of nucleic acid bases

was observed in the hippocampus and entorhinal cortex in AD.²¹ Nitrous acid, can also initiate protein (e.g., histone)-DNA crosslinks through oxidative modification of guanine residues, as well as^{23,24} DNA-DNA inter-strand crosslinks.²⁵

ROS and Amyloid- β

Amyloid- β may serve as a source of ROS, as it has been shown to bind to Cu^{2+} and Zn^{2+} , which also induce amyloid- β aggregation. In the presence of biological reducing agents, Cu^{2+} bound to amyloid- β is reduced to Cu^+ , which, in mediating Fenton-type reactions, can exacerbate oxidative stress.²⁶ Extensive mitochondrial DNA damage was observed when rat pheochromocytin(PC12) cells were exposed to amyloid- β , showing a direct correlation between oxidative stress and DNA damage.²⁷ However, it has been shown that copper binding to amyloid- β results in conformational changes that confer Cu/Zn SOD activity.²⁸ Thus amyloid- β may also function to maintain oxidative balance. Further, it can act as an antioxidant by sequestering free radicals.²⁹ Hence, therapeutic intervention aimed at disrupting the relationship between transition metals (e.g., Cu^{2+}) and amyloid- β is in doubt.³⁰ Much needs to be understood concerning the nature of amyloid- β -metal interactions in order to design effective therapeutics aimed at reducing amyloid- β -induced oxidative stress.

Mechanisms of DNA Damage by ROS/RNS

ROS and RNS can damage DNA by modifying nucleic acid bases. Most of the damage to the nucleic acids arises from $\cdot\text{OH}$ produced from the metal-catalyzed Fenton reaction.³¹ The hydroxyl radical, can modify guanine, forming 8-hydroxyguanine, which may then mispair with adenine (Fig. 3). The resulting mispairing produces GC to TA transversion mutations.^{32,33} Thymine similarly gives rise to 5-hydroxymethyluracil (not the sole product) upon reaction

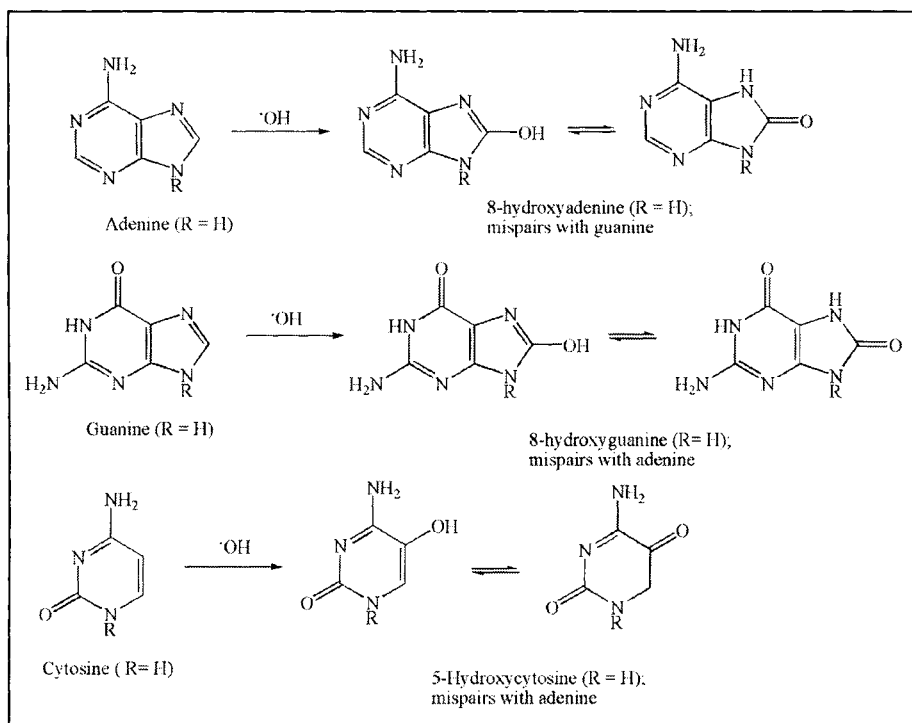


Figure 3. ROS induced hydroxylation of nucleic acid bases.

with ROS, which could mispair with guanine.²⁰ 8-Hydroxyadenine, a ROS-derived product of adenine, also could mispair with guanine.²⁰ 5-Hydroxycytosine could mispair with adenine giving CT transition mutations.³⁴

Reaction of ONOOH, like HNO_2 , with nucleic acid bases normally results in hydrolytic deamination, replacing NH_2 groups with OH groups. Thus adenine, cytosine and guanine are transformed into hypoxanthine, uracil, and xanthine, respectively (Fig. 4). Hypoxanthine mispairs with cytosine, and uracil mispairs with adenine.²⁰ A variety of oxidatively modified nucleic acid bases have been identified and measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Corresponding silylated derivatives can also be analyzed using gas chromatography-mass spectrometry (GC/MS) techniques. However, the potential for artifactual DNA damage, during isolation and sample preparation, requires that a number of sample work-up procedures, to minimize these limitations, must be considered.³⁵ On the other hand, immunocytochemical studies, using antibodies specific to known DNA modifications, are useful as they circumvent many of the problems associated with DNA extraction.³⁶ We feel that the best approach encompasses a combination of instrumental and in situ immunocytochemical studies in the study the DNA modifications induced by oxidative stress.

Nitrous/peroxynitrous acid induced deamination of nucleic acid bases occurs via the formation of the corresponding diazonium salts. Nucleophilic substitution of the diazonium group by water gives the corresponding oxygenated derivatives (e.g., guanine to xanthine, adenine to hypoxanthine, and cytosine to uracil) (Fig. 4). On the other hand, the diazonium group may also be substituted by the amino group of another nucleic acid base such as guanine to generate DNA crosslinking. Such interstrand DNA crosslinks at guanine sites, where two guanine residues are connected by a single exocyclic imino group, have been characterized (Fig. 5).^{25,37}

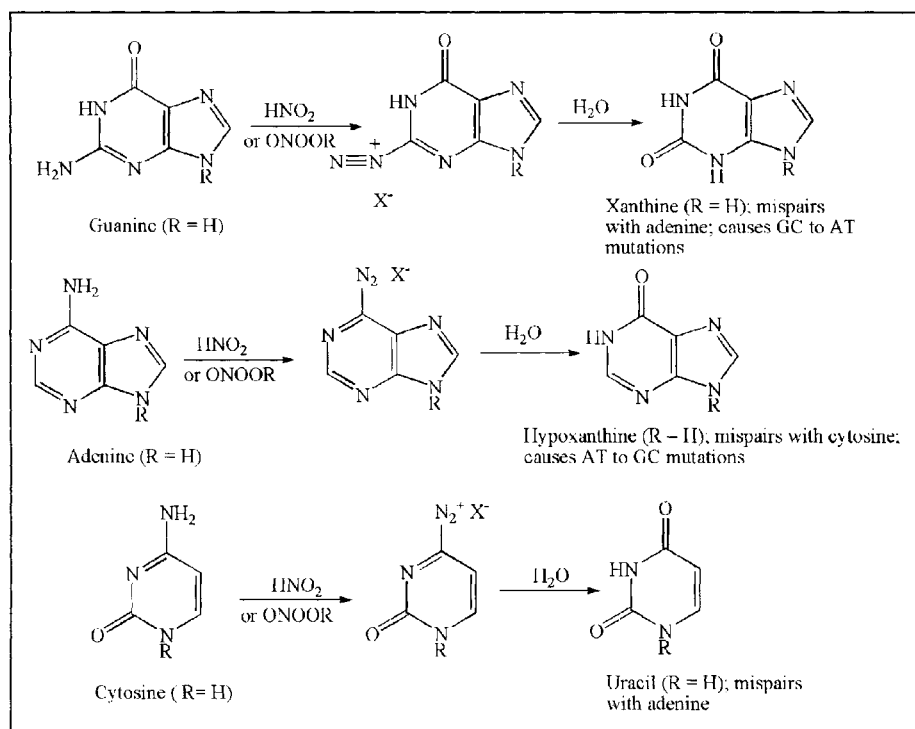


Figure 4. RNS induced hydroxylative deamination of nucleic acid bases.

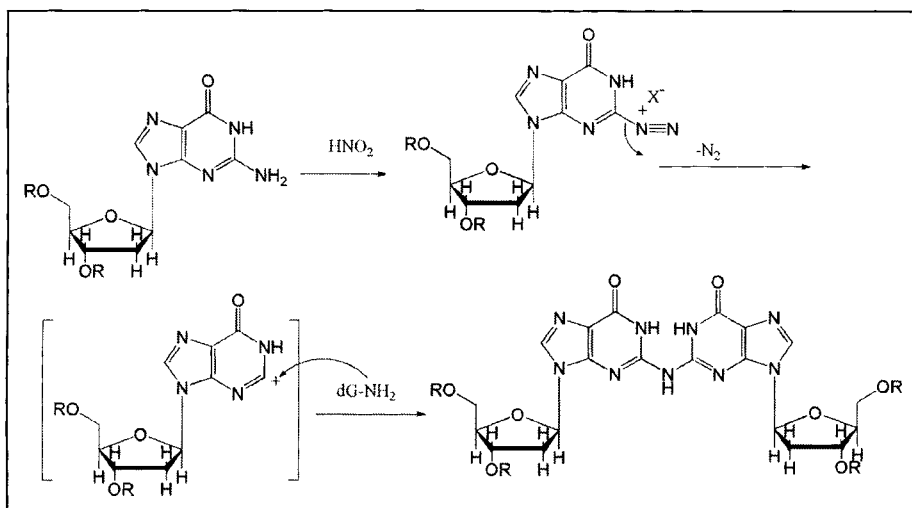


Figure 5. Interstrand crosslinking of DNA at guanine sites by nitrous acid.

Protection against ROS and RNS

Antioxidants, such as ascorbic acid, vitamin E, glutathione, and β -carotene can intercept superoxide and hydroperoxide radical anions as well as hydroxyl radicals, thereby limiting their toxic effects. In general, combinations of vitamin C and vitamin E are more effective as antioxidants compared to either one alone.³⁸ In these cases, vitamin E can be regenerated from its oxidatively modified form by reaction with vitamin C, which is relatively more abundant in vivo. CuZn-SOD and Mn-SOD react with superoxide radical anions and convert them into hydrogen peroxide and molecular oxygen. Downregulation of these enzymes can result in exacerbation of oxidative stress, although in AD brains they are abundant, ruling out the possibility that their deficiency results in the pathogenesis of AD.³⁹ Carnosine (β -alanylhistidine), a naturally occurring dipeptide, also provides some protection against ROS/RNS in AD.¹⁷

Oxidative Stress Involving Advanced Glycation End Products (AGEs) and Advanced Lipid Peroxidation End Products (ALEs)

AGEs and ALEs Products

Reactions of reducing sugars (e.g., ribose, glucose, ascorbate) with amino groups of proteins, followed by further oxidative modifications of the adducts leads to advanced glycation end products (AGEs), which contribute to the histopathological and biochemical hallmarks of AD. AGEs such as pentosidine (a cross-link between lysine and arginine), pyrraline (a lysine-derived adduct), and carboxymethyllysine (CML, the product of condensation of glyoxal with lysine) have been identified in elevated levels in AD brains.⁴⁰⁻⁴⁶ Carboxyethyllysine (CEL), an analogue of CML was found in other diseases such as diabetes, although it has yet to be observed in AD. Similarly, ALEs, formed by the reaction of proteins with lipid-peroxidation products (e.g., HNE), are elevated in AD (Fig. 6). HNE derived adducts of proteins were shown to be present in AD by immunocytochemical studies, using antibodies specific to various HNE adducts.⁴⁷⁻⁴⁹ Interestingly, antibodies specific for AGEs also cross-react with ALEs.⁵⁰

AGEs and ALEs can potentially act as further sources of ROS by their ability to chelate, and in some cases reduce, redox active transition metals. The AGEs also activate the receptor for AGE, indirectly contributing to an increased production of ROS.^{41,51} Glycation of amyloid- β markedly enhances its aggregation in vitro.⁵²

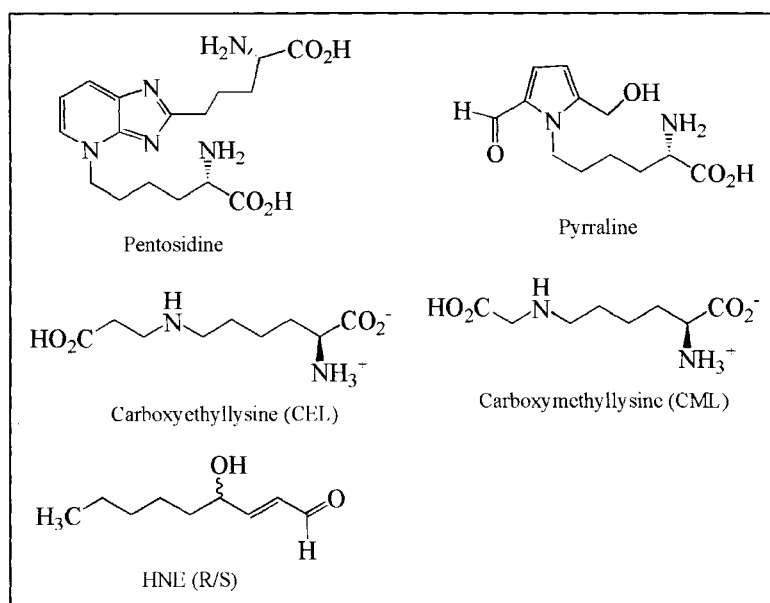


Figure 6. Structures of representative AGEs and HNE.

Several AGEs including CML and hydroimidazolones (derived from methylglyoxal or 3-deoxyglucosone) were found in relatively elevated amounts in the CSF of the subjects of AD, compared to those of the controls.⁵³ Thus AGEs, in addition to 3-nitrotyrosine (RNS modified proteins), may serve as important biomarkers for the onset of AD. CML is also elevated in CSF of patients with amyotrophic lateral sclerosis.⁵⁴

Microtubule associated τ -protein undergoes several posttranslational modifications including hyperphosphorylation and glycation and aggregates into paired helical filaments which upon crosslinking form NFTs, one of the hallmarks of AD.⁵⁵ Thus anti- and deglycating agents may serve as therapeutic agents in the prevention of the formation of NFTs. Recently carnosine was shown to be an antiglycating agent.^{17,56} It was found that histidine was more effective than carnosine as anti-crosslinking agent, whereas β -alanine showed relatively less anti-crosslinking properties than that of carnosine.⁵⁶ Thus the imidazolium group of histidine may stabilize the Maillard adducts formed through the reaction of the primary amino groups of carnosine or histidine and the reducing sugars.

DNA Damage Involving ALEs

Lipid peroxidation generates numerous cytotoxic aldehydes including acrolein, crotonaldehyde, HNE, and malondialdehyde (MDA). HNE, in particular, has been extensively explored in recent years in the pathogenesis of AD. It can form exocyclic 'propano- adducts by reaction with nucleoside purine and pyrimidine bases.^{16,57-59} Adducts involving the reaction of HNE with 2'-deoxyguanosine are found abundantly in human genomes, suggesting that the cellular defense system is not 100% efficient.⁶⁰ The reaction for the formation of HNE adduct presumably proceeds through the initial Michael addition of the free amino group (in the purine ring) to the HNE followed by nucleophilic addition of the N¹ (in the purine ring) to the carbonyl group (Fig. 7).

It has been shown that the 2'-deoxyguanosine/HNE propano adducts result in G to T transversion mutations in mammalian cells.⁶¹ The formation of HNE-DNA adducts results in the G to T transversion mutations predominantly at the third base of codon 249 of the p53

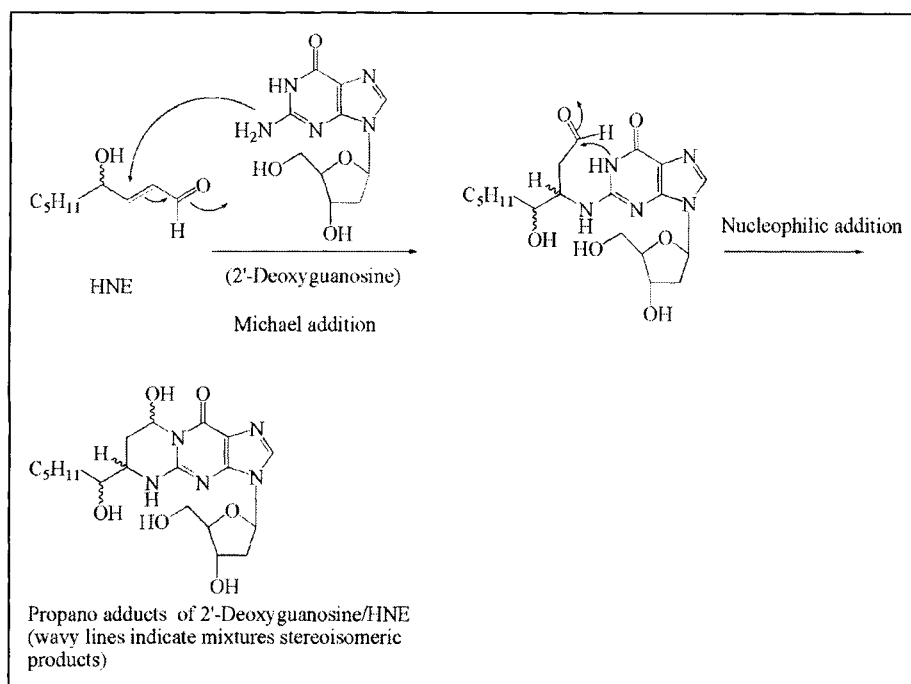


Figure 7. Formation of 2'-deoxyguanosine/HNE 'propano' adducts.

gene of human lymphoblastoid cells.⁶² The corresponding acrolein derived propano adducts are not as genotoxic as those of HNE, due to the availability of efficient repair mechanisms for the former adducts.⁶³ HNE derived propano adducts are highest in hippocampus, followed by the cerebellum and parietal cortex.¹⁶

4-Oxo-2-nonenal (4-ONE), the oxidized form of HNE^{64,65} is also shown to be involved in DNA damage.^{64,66-70} DNA forms exocyclic five-membered 'etheno-' adducts with 4-ONE through reactions with purine and pyrimidine rings. Several DNA/4-ONE adducts derived from 2'-deoxyadenosine, 2'-deoxyguanosine, and 2'-deoxycytosine have been characterized using atmospheric pressure ionization/MS/MS techniques⁶⁶ (Fig. 8). The formation of these etheno-adducts was postulated to involve initial nucleophilic addition of the free amino group of the purine/pyrimidine rings to the carbonyl group of 4-ONE, followed by intramolecular (cyclizing) Michael addition and subsequent dehydration.⁶⁶ The etheno- adducts are formed in high yields when 2'-deoxyguanosine and 2'-deoxyadenosine are treated with 4-ONE.⁶⁴ Although similar etheno- adducts can also be formed from the reaction of 2,3-epoxy-4-hydroxynonenal with DNA, the latter epoxide is unlikely to be formed *in vivo* from HNE.⁷¹ Cultured human monocytes incubated with HNE showed over 95% of the HNE-2'-deoxyguanosine propano adducts among the overall adducts of DNA. The other minor products included 1,N⁶-ethenoguanine and 1,N⁶-ethenoadenine.⁷²

4-ONE is more reactive and cytotoxic than HNE,⁷³ and may be at least as biologically important. Its adduct with vitamin C has been recently found in human plasma.⁷⁴ 4-ONE also readily modifies proteins,⁷⁵ and it could interact with proteins responsible for nucleotide excision repair, damaging DNA repair mechanisms.

The effects of HNE on DNA damage and inhibition of DNA repair has been explored in human cells in case of mutations responsible for cancer,⁷⁶ although there has been no such evidence of HNE modification of DNA repair enzymes in AD. In the absence of appropriate DNA repair mechanisms, the DNA adducts can induce apoptosis of neuronal cells.⁷⁷

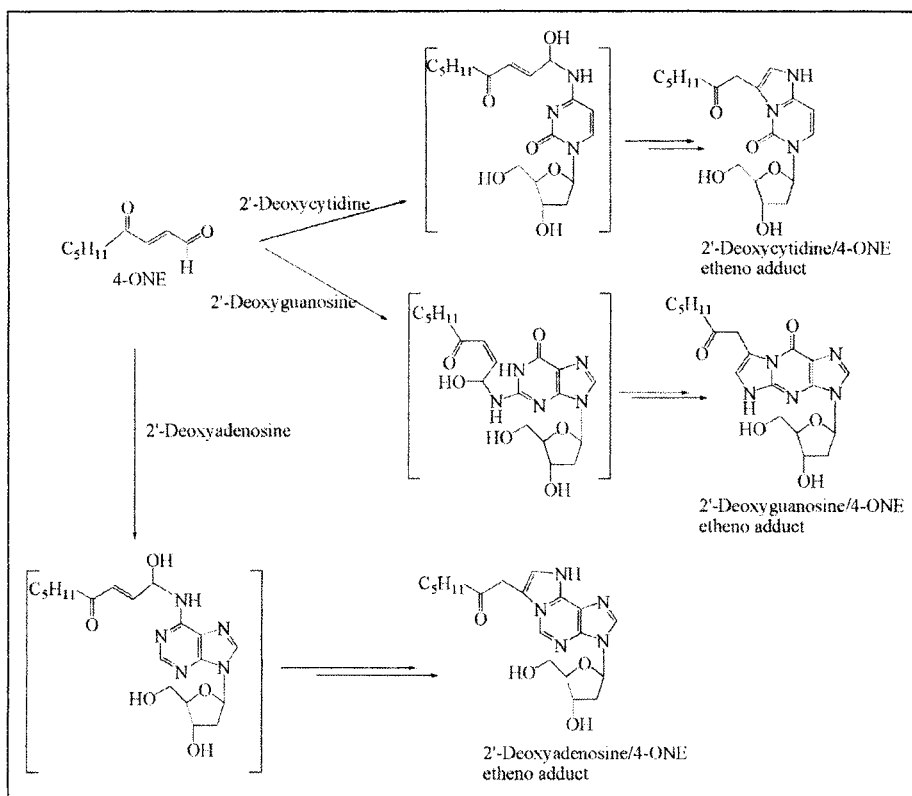


Figure 8. 4-ONE induced DNA damage forming 'etheno' adducts.

HNE Detoxification

HNE and other lipid peroxidation derived α,β -unsaturated aldehydes (e.g., acrolein) can be detoxified by anti-inflammatory agents such as carnosine through Michael adduct formation.^{78,79} Similarly, the glutathione transferase (GSH-transferase) superfamily of enzymes catalyzes the Michael type reaction of glutathione (GSH) with HNE (Fig. 9).⁸⁰ These Michael adducts are relatively nontoxic, compared to HNE. In another study it has been shown that depletion of intracellular glutathione levels enhances the formation of endogenous cyclic DNA adducts derived from HNE in rat liver.⁸¹

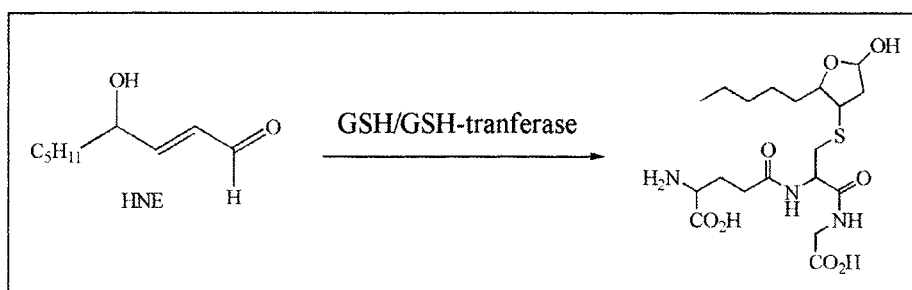


Figure 9. HNE detoxification through Michael additions of GSH.

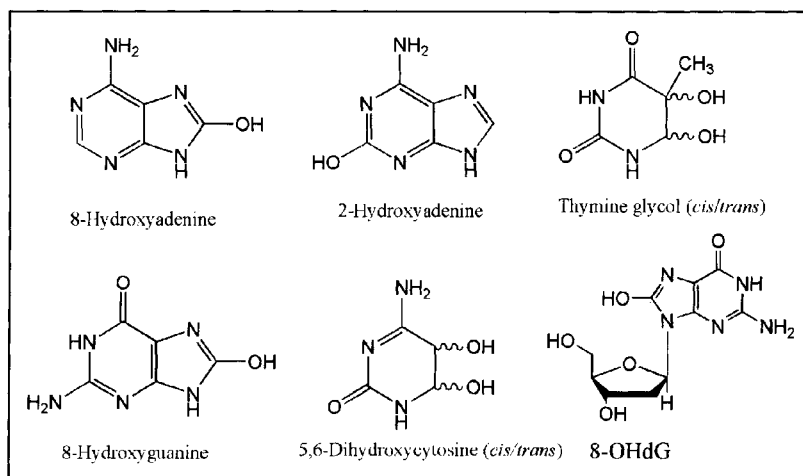


Figure 10. Structures of some of the oxidatively modified nucleic acid bases and 8-OHdG.

Markers of Nucleic Acid Damage in AD

Reaction of hydroxyl radicals with purine and pyrimidine nucleic acid bases gives multiple products⁸² (Fig. 10). Hydroxyl radicals can react with adenine and guanine at C₄, C₅, or C₈ positions in the pyrimidine ring. Thymine can undergo the addition of hydroxyl radicals at C₄ or C₅ positions to give the corresponding radicals, which upon reaction with O₂ give peroxide products. Following reductive cleavage, the latter peroxides can give *cis*- and *trans*-thymine glycols. Thymine can also undergo hydrogen abstraction from the methyl group to give the corresponding free radical which, upon reaction with O₂, followed by reductive cleavage gives the corresponding alcohol. Cytosine similarly can give several products including cytosine glycol and 5,6-dihydroxycytosine.

Although over 20 different oxidation products of DNA have been characterized, most of the investigators have focused attention on 8-hydroxyguanine, or its deoxynucleoside equivalent, 8-OHdG, as a marker of DNA damage arising from oxidative stress. Oxidative damage of DNA involving hydroxyl radicals, produced by the hypoxanthine/xanthine oxidase system in the presence of Fe²⁺/Fe³⁺ ions, results in the modification of the purine and pyrimidine residues of nucleic acid bases.⁸³ Using GC/MS, the following base products were observed after acidic hydrolysis of the damaged DNA: cytosine glycol, 5,6-dihydroxycytosine, 4,6-diamino-5-formamidopyrimidine (Fapy-adenine), 8-hydroxyadenine, 2,6-diamino-4-hydroxy-5-formamidopyrimidine (Fapy-guanine), and 8-hydroxyguanine (Fig. 11). Of these products, Fapy-guanine was the major product, followed by 8-hydroxyguanine, for DNA treated with hypoxanthine/xanthine oxidase/Fe³⁺-EDTA.⁸³

8-Hydroxyguanine and Fapy-guanine were shown to be the major products of DNA oxidation by ROS and RNS.⁸⁴ Using GC/MS techniques, Halliwell and colleagues identified several oxidized products of DNA bases, including 8-hydroxyadenine, 8-hydroxyguanine, thymine glycol, Fapy-guanine, 5-hydroxyuracil, and Fapy-adenine in parietal, temporal, occipital, and frontal lobe, superior temporal gyrus, and hippocampus of AD brains,⁸⁵ implying a role for such DNA damage in the pathogenesis of AD.

Elevated levels of 8-OHdG, 8-hydroxyadenine, and 5-hydroxyuracil, a product of cytosine oxidation, were observed from the nuclear DNA (nDNA) of parietal, temporal and frontal lobes of AD brains as compared with age-matched controls,⁸⁶ although there was no significant correlation between the oxidized bases and neurofibrillary tangles and senile plaque counts. The levels of 8-OHdG were significantly elevated in intact DNA isolated from ventricular CSF of AD subjects. However, the corresponding levels of the free 8-OHdG, formed as a result of

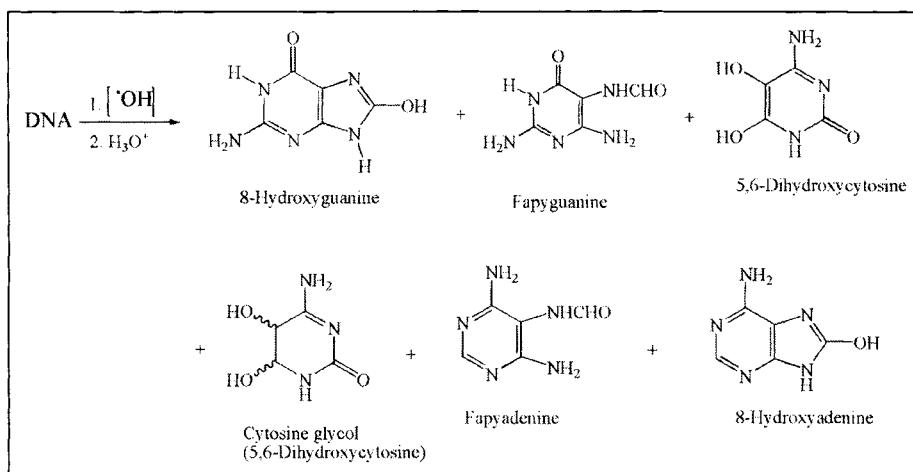


Figure 11. Products of hydroxyl radical mediated oxidative DNA damage, followed by aqueous hydrolysis.

repair mechanisms, were significantly lower in CSF, implying deficiencies in the repair mechanisms for the removal of oxidized bases from the damaged DNA.⁸⁷ These observations show that DNA damage is not only greater in AD, but also DNA repair mechanisms may be impaired in AD. Using the Comet assay with oxidative lesion-specific DNA repair endonucleases (endonuclease III for oxidized pyrimidines, Fapy-glycosylase for oxidized purines), it has been shown that AD is associated with elevated levels of 8-OHdG and other oxidized nucleic acids.⁸⁸

Lovell and coworkers have found statistically significant decreases in the activity of 8-OHdG glycosylase, an enzyme responsible for the excision of 8-OHdG, in the nuclear fraction of AD hippocampal and parahippocampal gyri superior and middle temporal gyri, and inferior parietal lobule (IPL). Further, depletion of the helicase activity was also observed in the nuclear fraction in the IPL of AD.⁸⁹ Thus, it is likely that the decreased repair of DNA damage could lead to the pathogenesis of AD.

Smith and colleagues investigated the formation of 8-OHdG and 8-hydroxyguanosine (8-OHGuo) in AD using immunoreactivity with monoclonal antibodies 1F7 and 15A3 specific to 8-OHdG and 8-OHGuo.⁹⁰ From these studies it was found that 8-OHdG and 8-OHGuo were prominent in the cytoplasm, and to a lesser extent in the nucleolus and nuclear envelope in neurons within the hippocampus, subiculum, and entorhinal cortex as well as frontal, temporal, and occipital neocortex in AD. Control cases were only faintly immunolabeled in similar structures. Damage to RNA is likely to be more extensive than to DNA, as there are more single-stranded regions in RNA, compared to DNA, and a lack of protective histones.⁸ Furthermore, it is plausible that the metals bound to RNA are major sites of redox activity, which result in the formation of ROS. The oxidized bases in AD are associated predominantly with RNA, as immunoreactivity towards 8-OHGuo was diminished greatly by preincubation with RNase but only slightly by DNase.¹¹ A recent paper has suggested that mitochondrial ROS damage mitochondrial DNA, but not nuclear DNA.⁹¹ It was concluded that the ROS derived from the mitochondrial respiratory chain are detoxified by mitochondrial SOD and other agents such as cytosolic catalase, and thus are not able to travel the distance from the mitochondria to the nucleus.

Quantitatively, neuronal 8-OHGuo is greatest early in AD and is reduced with disease progression. Interestingly, nitrotyrosine, a marker of RNS mediated oxidative stress, is also elevated in the early stages of AD and is reduced with disease progression, indicating the significance of RNS in RNA damage in AD (vide infra). Smith and coworkers observed that the increase in

amyloid- β deposition is associated with decreased oxidative damage.¹⁰ Furthermore, neurons with neurofibrillary tangles show a 40% to 56% decrease in 8-OHGuo levels compared with neurons free of neurofibrillary tangles, demonstrating that oxidative stress-induced RNA and DNA damage is an early event in AD, that decreases with disease progression.¹⁰ Thus in AD, increased levels of oxidative damage to DNA occurs prior to the onset of amyloid- β deposition. As markers of ALE-induced DNA damage, 'etheno-' and 'propano-' adducts are commonly used (*vide supra*). Their characterization could be achieved through LC/MS-MS.⁹²

Mitochondrial DNA Damage

8-OHdG, a marker of DNA damage, is present in significant amounts (*i.e.*, about three-fold increase as compared to control cases) in the mitochondrial DNA (mtDNA) as well as in nDNA of AD brains.⁹³ However, mtDNA is more prone to oxidative damage as compared to that of nDNA, as the latter is well protected by histones and other DNA-binding proteins.⁹⁴ The mtDNA is also exposed to increased concentrations of ROS. The oxidative stress involving mtDNA results in deleterious DNA-DNA and DNA-protein crosslinking, in addition to transversion mutations.^{95,96}

A significant reduction of mitochondrial membrane fluidity was observed in AD along with increased levels of 8-OHdG in mtDNA. The alteration in membrane fluidity is primarily a result of lipid peroxidation. HNE and malondialdehyde (MDA), two widely studied lipid peroxidation products, were isolated in mitochondria, and more importantly, HNE was shown to modify the membrane fluidity by direct interaction with membrane phospholipids.⁹⁷ Thus, there is a direct correlation between oxidative stress and DNA damage, implicating oxidative stress in the pathogenesis of AD.⁹⁸

In aging brains, there is substantial oxidative damage to mtDNA, which may be due to impaired mitochondrial function resulting in increased ROS, or by reducing ATP required for DNA repair,⁹⁹ a major risk factor for the onset of AD. The levels of 8-OHdG in DNA isolated from three regions of cerebral cortex and cerebellum increase progressively with normal aging in both nDNA and mtDNA.⁹⁸ The rate of increase of 8-OHdG, however, was 10-15 times higher in mtDNA than nDNA.^{98,100} The relatively higher mtDNA damage may be explained as due to the absence of protective histones and the abundance of ROS in mitochondria.³⁴ 8-OHdG is commonly used as a biomarker for DNA damage as it is approximately 10-fold higher than other base adducts. It was shown that DNA from the temporal lobe of AD cases showed the most oxidative damage, whereas cerebellum in those cases was only slightly affected, suggesting that oxidative damage to mtDNA may contribute to the neurodegeneration of AD.⁹⁵

The ROS and RNS generated as a result of impaired mitochondrial function, combined with reduced ATP levels in these mitochondria, can contribute to damage of vulnerable genes in the aging human brain.⁹⁹ However, it is not clear whether this would have a significant effect on total cellular energy metabolism: as there are thousands of mitochondria, it is unlikely that DNA damage may occur in the same gene in all of them.^{101,102} However, when mutations occur in mtDNA, the corresponding mutant proteins may increase the inefficiency of the mitochondrial respiratory chain, resulting in the excessive production of ROS that cause further mtDNA and nDNA damage.

DNA Repair in AD

Cells with damaged DNA can undergo apoptosis through a common p53-dependent mechanism.¹⁰³ However, DNA damage can be repaired by a combination of enzymes. If DNA damage is not repaired, it may be bypassed by specialized DNA polymerases.¹⁰⁴ The repair of DNA typically involves several steps. Excision enzymes such as DNA glycosylases remove damaged bases by the cleavage of the base-sugar bonds. An endonuclease nicks the DNA strand at this site and removes it. Then, DNA polymerase fills the missing link in the strand, and a DNA ligase joins this new DNA strand with the existing undamaged strand.²⁰ In addition to base excision repair, nucleotide excision repair may also be involved. The nucleotide excision

repair involves removal of damaged nucleotides as part of large (~30 nucleotide units) fragments.¹⁰⁵ Bcl-2, an integral membrane protein may facilitate DNA repair after oxidative stress as recovery of DNA is accelerated in cells expressing Bcl-2 protein.¹⁰⁶ A growth arrest DNA damage-inducible protein, GADD45, is expressed in AD neurons and is associated with expression of Bcl-2.¹⁰⁷ In addition to these, Mre11 protein complex¹⁰⁸ and poly(ADP-ribose) polymerase (PARP) enzymes¹⁰⁹ may also contribute to DNA repair.

The accumulation of the damaged DNA bases in cells may result in the loss of normal cellular function, which could be a causative factor in AD and other age related diseases. In addition, pyrimidines next to the 8-OHdG residue are also misread¹¹⁰ during DNA transcription.

Nucleotide/Base Excision Repair

Base excision repair (BER) and nucleotide excision repair (NER) of DNA gives 8-hydroxyguanine (8-OHGu) and 8-OHdG, respectively, as the products that are excreted into CSF, blood plasma, and eventually to urine (although the latter is not proven). Quantification of these species indicates the extent of oxidative DNA damage.^{111,112} By far BER seems to be the predominant mechanism for the repair of the oxidatively modified DNA. It was found that the concentrations of 8-OHdG in CSF of AD are 100-fold higher than those of healthy individuals. In addition, the ratio of 8-OHdG to 8-hydroxyguanine is approximately 8-fold higher in CSF than in urine, suggesting that the nucleotide excision repair is a major DNA repair mechanism in removal of oxidatively damaged DNA in brain cells.¹¹³ NER requires the TFIIH transcription-repair complex having helicase activity. These DNA helicases unwind DNA for repair as well as for replication. BER, NER, and mismatch repair have been identified and characterized in eukaryotes.¹⁰⁵ In addition to these, a variety of other protein complexes are also involved in DNA repair.

There are a variety of base-specific glycosylases that cleave specific damaged bases.¹¹⁴ For example, human HeLa cell extracts contain two forms of 8-hydroxyguanine glycosylase (hOGG) repair enzymes. hOGG-1 cleaves 8-hydroxyguanine paired with cytosine or thymine, whereas hOGG-2 cleaves 8-hydroxyguanine paired with adenine. Expression of DNA excision repair cross complementing proteins p80 and p89 was observed in AD brains.¹¹⁵ These are known to repair different types of DNA damage.

8-Oxoguanine DNA glycosylase (hOGG1-2a) is one of the excision repair enzymes that repairs 8-OHdG. Using an antibody specific to the mitochondrial form of hOGG1-2a, it was found that hOGG1-2a is expressed mainly in the neuronal cytoplasm in both AD and control cases in regionally different manners.¹¹⁶ Immunoreactivity against hOGG1-2a is associated with neurofibrillary tangles, dystrophic neurites and reactive astrocytes in AD. The relatively low levels of hOGG1-2a expression in AD indicates that oxidative DNA damage in mitochondria may be involved as a pathogenic factor.¹¹⁶ Another repair enzyme for oxidative DNA damage, purine-nucleoside triphosphatase (hMTH1) was also observed in neurons of AD. In vitro studies showed that hOGG1-2a immunoreactivities in reactive astrocytes and oligodendrocytes were more intense than those to hMTH1.¹¹⁷ By adding H₂O₂ to the cultured astrocyte cells, rapid induction of hMTH1 was observed, whereas the levels of hOGG1-2a were mildly increased, showing that hMTH1 is an inducible enzyme under oxidative stress, and hOGG1-2a is rather constitutively expressed and also up-regulated in the chronic stage of the disease.¹¹⁷

Conclusions

Oxidative stress has a marked impact on the pathogenesis of AD. ROS and RNS modify nucleic acid bases, a process which results in transversion mutations. DNA is also modified by AGEs and ALEs which by themselves are produced through the involvement of ROS and RNS. Cellular antioxidant defenses attenuate the effects of ROS and RNS. However, when there is an imbalance between the production of ROS/RNS and cellular antioxidant defense mechanisms, oxidative stress results, causing DNA damage and modification of other biomacromolecules such as proteins and lipids. DNA damage is repaired by a variety of pathways, although mostly through BER and NER mechanisms.

Acknowledgements

Work in the authors' laboratories is supported by the NIH (NS38648 and AG14249) and the Alzheimer's Association (IIRG-03-6263 and IIRG-04-1272).

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CHAPTER 11

Nucleic Acid Oxidation and the Pathogenesis of Cardiovascular Diseases

Maria Grazia Andreassi*

Abstract

Cardiovascular disease is the dominant health problem in the western world. The most frequent underlying cause of cardiovascular disease is atherosclerosis. The cellular and molecular mechanisms involved in atherosclerosis and its acute complications are being defined, but much is still unknown. Growing evidence indicates that oxidative damage to nuclear and mitochondrial DNA may represent an important link between the inflammatory nature and the oxidative theory of atherosclerosis. Various animal models of atherosclerosis support the evidence that oxidatively damaged DNA plays a key role in both the formation and the complications of atherosclerosis. Human investigations also support a mutational hypothesis of atherosclerosis. Future research on the mechanism by which oxidatively damaged DNA participates in the atherogenic process may provide new insights for early diagnosis and treatment of atherosclerosis.

Introduction

The American Heart Association reports that cardiovascular disease is the leading cause of death in the United States and the cause of more than half of all mortality in the world's developed countries.¹ Major clinical manifestations of cardiovascular disease include myocardial infarction, coronary artery disease, stroke, peripheral artery disease and congestive heart failure. In most cases, these clinical conditions result from atherosclerosis, a progressive disease of the arterial wall, characterized by focal thickening and luminal obstruction.² The old view, not more than 10 to 15 years ago, saw atherosclerosis as a simple 'plumbing problem' due to a gradual build-up of a plaque containing cholesterol and fatty material on the surface of the passive artery walls. Currently, new views recognize atherosclerosis as a chronic inflammatory disease as it has been defined by Ross in 1999,³ involving many cell interactions at the level of the active vascular wall. This view initiated a complete shift in the atherosclerosis research field and it has focused attention on the cellular and molecular understanding of vascular function. In fact, understanding the basic pathophysiological mechanism of atherosclerosis is essential in order to improve both the prevention and the treatment of the majority of cardiovascular diseases. Actually, there has been growing interest in the role of oxidative DNA damage as a further mechanism involved in atherosclerotic plaque development and its complications.⁴⁻¹⁰ The purpose of this chapter is to explore the evidence for the presence and the potential pathogenic role of oxidative DNA damage in the atherogenic process.

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Pathogenesis of Atherosclerosis

Atherosclerosis is a chronic condition which begins with the "fatty streak" lesions in the artery walls, due to the accumulation of lipids in macrophages. Arterial depositions of fat may be observed in the coronary arteries even during the first decade of an individual's life.¹¹ This evidence supports the chronic character of human atherosclerosis and leads to the proposition that the disease is part of the general process of aging. The disease progresses through several stages, ultimately ending with a complex plaque accumulated in the artery wall, which blocks blood flow and can precipitate acute clinical manifestations. Acute clinical events, such as myocardial infarction or stroke, are often the result of rupture or ulceration of an "unstable" or "vulnerable" plaque.¹²

Although several histo-morphological features indicate which plaques are at risk of rupture, the triggering factors responsible for plaque rupture are not well defined.¹³ In particular, very little is known about the molecular mechanism through which the plaque is disrupted or about the relationship between plaque disruption and both the trigger and onset of acute disease. At least three hypotheses have been proposed in order to explain the origin of atherosclerotic disease. Although every hypothesis concentrates on a particular aspect of the atherosclerotic process, extensive overlap exists.

Inflammatory—Response to Injury Hypothesis

The central hypothesis concerning the pathogenesis of atherosclerosis is the "inflammatory response to injury" hypothesis.³ This hypothesis postulates that atherosclerotic lesions represent an inflammatory-fibroproliferative response to different insults (e.g., cigarette smoking, hypertension, diabetes and infectious agents) to the endothelium. The endothelial dysfunction induces the recruitment of monocytes, macrophages and T lymphocytes from the bloodstream that interact with cellular adhesion molecules and migrate through the endothelium, situating themselves within the sub-endothelial layer. The earliest pathologic lesion of atherosclerosis is the fatty streak, where the macrophages accumulate lipids and become large foam cells which, in turn, release growth factors and cytokines that continue to stimulate the inflammatory response and promote the migration of smooth muscle cells (SMCs). SMCs are responsible for the deposition of extracellular connective tissue matrix and form a fibrous cap that overlies a core of lipid-laden foam cells, extracellular lipid, and dying or dead cells (including apoptotic cells). Acute ischemic events are due to "unstable" plaques prone to fibrous cap rupture or plaque surface erosion, which trigger thrombus formation and vessel lumen occlusion, rather than lumen stenosis stemming from exaggerated plaque growth (Fig. 1).

Inflammation is believed to be a major factor in plaque rupture, and susceptible plaques tend to have a damaged fibrous cap and numerous inflammatory cells in residence.¹⁴ In particular, macrophages seem to play a prominent role in plaque destabilization since they produce many inflammatory mediators that contribute to the disruption of the vulnerable plaque.¹⁴ Despite our progress in understanding the inflammatory process of atherosclerosis, the inflammatory response-to-injury hypothesis sheds little light on the observation that proliferation of SMCs is a monoclonal process.

Monoclonal Hypothesis

The "monoclonal hypothesis" of atherosclerosis, originally proposed by Benditt and Benditt,¹⁵ states that atherosclerosis is a benign tumour of the artery wall. In this theory, SMC proliferation in an atherosclerotic lesion begins as a mutation or viral hit, transforming a single, isolated cell into the progenitor of a proliferative clone (Fig. 2). The original observation for this theory came from a study conducted on women with a genetic deficiency of glucose-6 phosphate dehydrogenase in which 80% of atherosclerotic plaques were found to be monoclonal.¹⁵ Another study reported that 89.7% of fibrous plaques were monoclonal, whereas only 17.8% of fatty streaks were monoclonal.¹⁶ In more recent studies of PCR amplification of the DNA of an X-inactivated gene, the monoclonal origin of the SMC was confirmed, but the relevance of monoclonality to plaque aetiology remains unclear.¹⁷⁻¹⁹

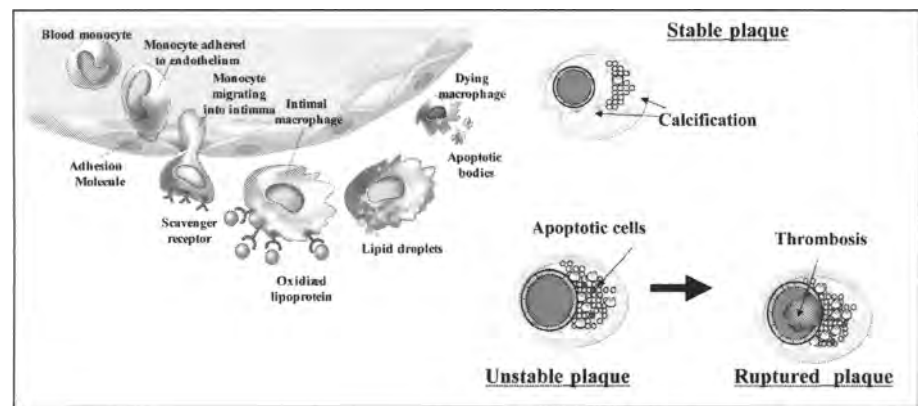


Figure 1. Overview of inflammatory-response to injury hypothesis. In early atherogenesis, endothelial dysfunction induces the recruitment of inflammatory cells and the accumulation of lipids (foam cells) leading to formation of an atherosclerotic plaque. Atherosclerotic lesions can be stable or unstable. Calcium deposits might prevent plaque disruption. Vulnerable plaques or unstable plaques have increased numbers of inflammatory cells, mostly macrophages, or dead or dying cells (derived from macrophages and SMCs). Inflammatory mediators produced by macrophages seem to be central in the disruption of the vulnerable plaques that trigger thrombosis. (Adapted from Libby, 2002.)¹⁴

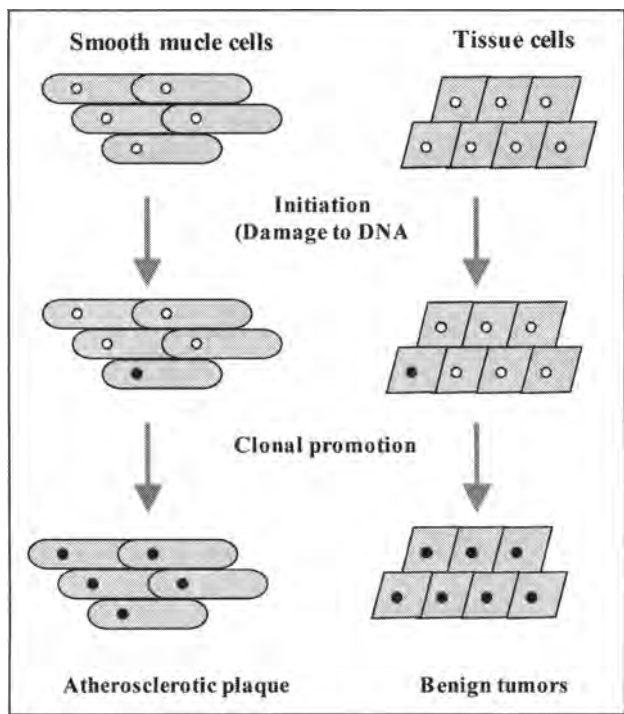


Figure 2. Overview of monoclonal hypothesis. Atherosclerosis begins as a mutation or viral hit, transforming a single, isolated smooth muscle cell into the progenitor of a proliferative clone, akin to the process of human cancer development.

Oxidative Hypothesis

Oxidative stress and the production of reactive oxygen species (ROS) are key elements in the progression of atherosclerosis.^{20,21} The “oxidative hypothesis” of atherosclerosis states that the oxidative modification of low-density lipoproteins (LDLs, the main carrier of lipids and cholesterol in the body) is central to the atherogenic process (Fig. 3).^{22,23}

The mechanisms by which oxidized LDLs and oxidative stress actually contribute to the disease progression are topics of intense research.^{22,23} Oxidized LDLs, rather than unmodified LDLs, are proposed to accelerate atherogenesis by different mechanisms, including interaction with “scavenger” receptors on the surface of macrophages that induces the formation of “foam” cells. Oxidized LDL also exhibits cytotoxic, chemo-attractant and cell growth stimulating activities.²⁴ ROS, in addition to the mechanism described above, modulate important signal transduction pathways that regulate vascular cell function and stimulate SMC proliferation.^{21,25}

Oxidative stress is also known to be an important cause of genetic alterations, including oxidative modification of DNA, micro-satellite and chromosomal instability.²⁶⁻²⁸ Therefore,

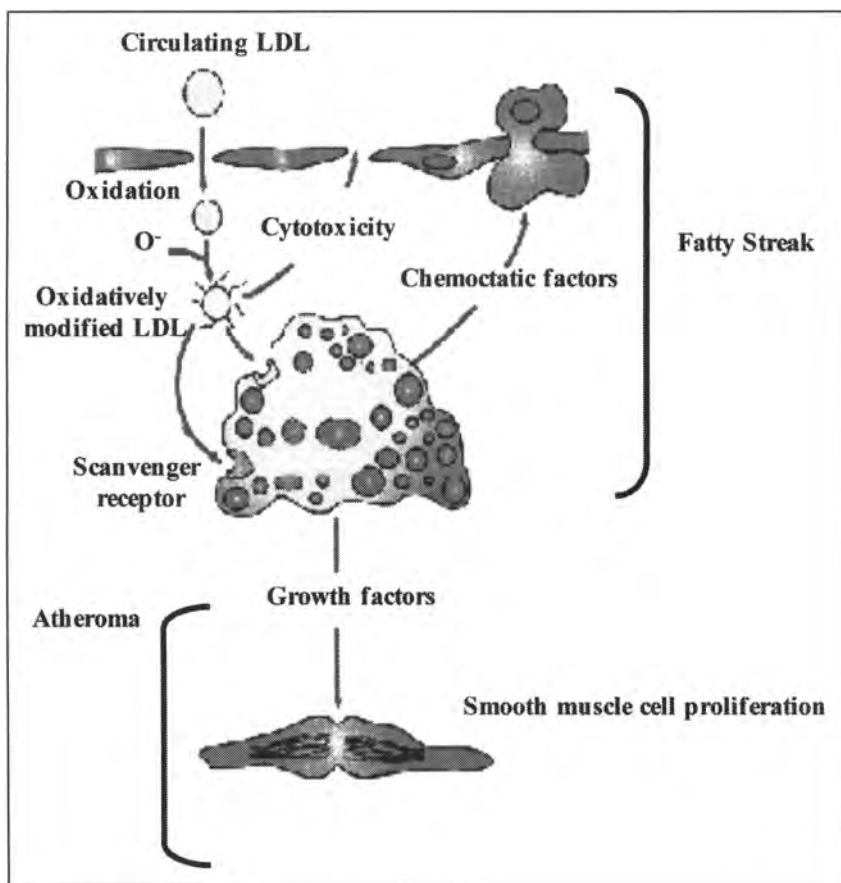


Figure 3. Overview of oxidative hypothesis. The passage and the oxidation of LDL across the endothelium into the artery wall is necessary for the formation of fatty streak. This process leads to the recruitment of circulating monocytes and T lymphocytes, the formation of foam cells from macrophages that bind oxidatively LDL via scavenger receptors, the secretion of growth factors that induce SMC migration into the intima. (Adapted from Andreassi, 2003.)⁷

oxidatively damaged DNA could be another important factor by which oxidative stress contributes to atherosclerosis,^{4,7} representing a potential unifying mechanism across the main theories of atherogenesis.

Genetic Instability and Oxidatively Damaged DNA in Atherosclerosis

In support of the mutational hypothesis of atherosclerosis, induced by oxidative stress, a diverse body of literature has identified loss of heterozygosity, modifications of DNA, and chromosome alterations in human atherosclerotic plaques.^{7,8}

Loss of Heterozygosity and Microsatellite Instability

The detection of loss of heterozygosity (LOH) and microsatellite instability (MI) in DNA extracted from atherosclerotic plaques, compared to DNA extracted from adjacent normal tissue, has been reported.³⁰⁻³⁷ The available evidence suggests that LOH and MI of specific genes could be an important molecular mechanism associated to the atherogenic process.³⁰⁻³⁷

Of special interest, the study by McCaffrey et al³² was the first demonstration of a frequent somatic cell mutation in atherosclerosis, supporting the hypothesis that "arteriosclerosis is just another form of cancer".³⁸ Specifically, these investigators reported that MI in the gene coding for the type II transforming growth factor- β 1 (TGF- β 1) receptor leads to premature truncation of the receptor transcript, with a consequent defective receptor structure. This causes a resistance to the anti-proliferative and apoptotic effects of TGF- β 1. However, this observation has not been supported by a subsequent report that found the presence of the mutation in the type II TGF- β 1 receptor only at low frequency in early to advanced atherosclerotic lesions.³⁶ Recently, significant genomic rearrangements have been identified on chromosomal arms 1p32-p31, 1q22-q25, 2q35 and 6p21.3 (Fig. 4), where genes involved in leukocyte adhesion, SMC growth, differentiation and migration (e.g., VCAM1, SELE, APEG1 and AIF1) have

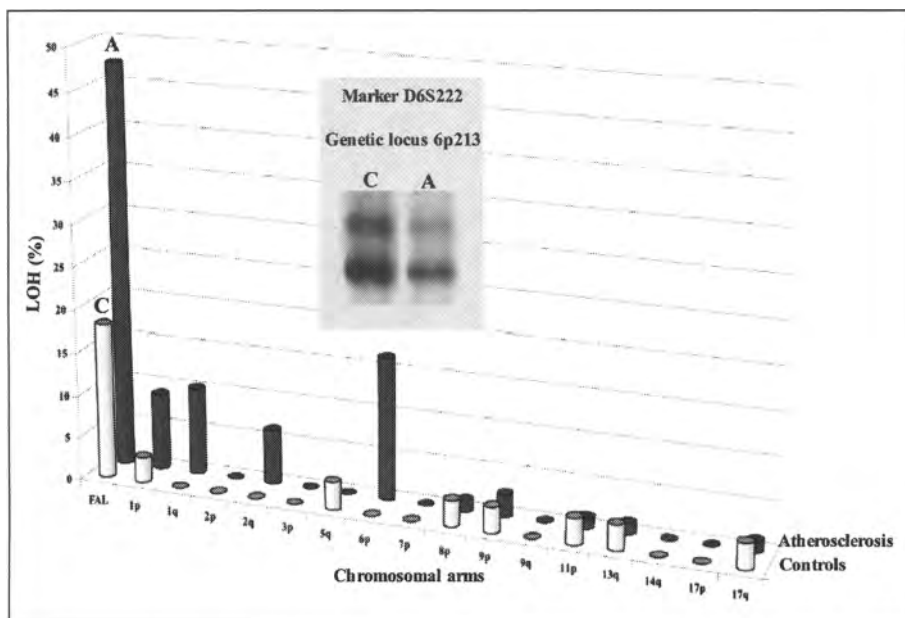


Figure 4. Loss of heterozygosity (LOH) incidence for DNA microsatellite markers tested for genetic locus in atherosclerotic and normal aortic specimens. (Adapted from Arvanitis et al, 2005.)³⁷

been mapped.³⁷ These findings suggest an important link between somatic DNA mutations and the inflammatory nature of atherosclerosis, providing a unified perspective of disease development.³⁷

Chromosomal Alterations

A limited number of studies have tried to investigate the presence of chromosomal aberrations in atherosclerotic plaques. Single or associated clonal chromosomal abnormalities were found in primary cell cultures from human atherosclerotic plaques.^{39,40} These mainly involved loss of the Y chromosome and several trisomies (XXY, chromosomes 10, 18, and trisomy 7).

More recently, Matturri et al⁴¹ showed that unstable atherosclerotic plaques presented a variety of chromosomal abnormalities in carotid endarterectomy specimens. Conversely, stable plaques did not present any chromosomal abnormalities, supporting the hypothesis that genetic instability might be of particular importance in the mechanisms of plaque evolution. It is also interesting to note that chromosome 7 trisomy was the most constant and significant in terms of chromosomal alterations in these studies (Fig. 5).³⁹⁻⁴² Chromosome 7 includes the gene for the A-chain of platelet-derived growth factor, the MET-proto-oncogene, and receptor gene for epidermal growth factor, and the finding of trisomy 7 in SMC of an unstable plaque would suggest the possible transformation of an advanced atherosclerotic plaque via a neoplastic-like process.³⁹⁻⁴²

Aromatic and Oxidative DNA Adducts

Evidence for the presence of both aromatic and/or oxidatively modified lesions in cardiovascular tissue has also been obtained from experimental animal models and clinical investigations. Preliminary animal studies have shown high levels of DNA adducts in heart and aorta, as well as lung, after exposure of rats to a mixture of side stream smoke and mainstream smoke.⁴³

Similar DNA adducts also occur in SMCs of human abdominal aorta affected by atherosclerosis, and their levels are significantly correlated with known atherogenic risk factors including age, number of currently smoked cigarettes, arterial pressure, blood cholesterol and triglycerides.⁴⁴ Such evidence has been supported by a study evaluating DNA adduct levels in patients undergoing open heart surgery.⁴⁵ Patients suffering from severe coronary artery disease tended to have higher adduct levels in their heart tissue than those having no or mild coronary artery disease.⁴⁵ Moreover, the levels of DNA adducts were highly related to smoking habits (Fig. 6).

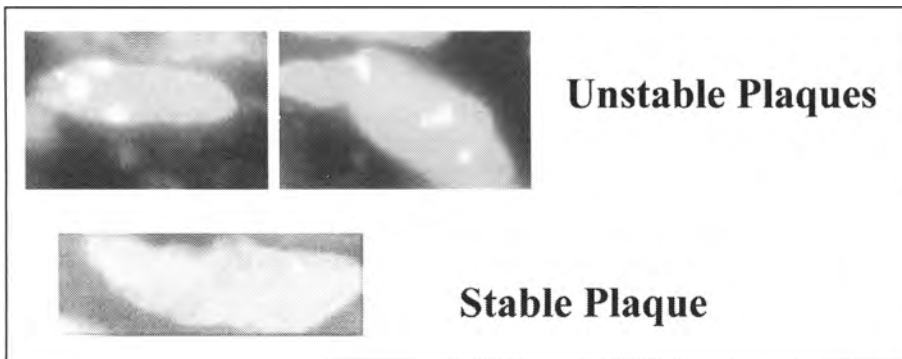


Figure 5. Fluorescence in situ hybridization with a probe for chromosome 7 on paraffin-embedded section of human atherosclerotic plaque. The cell nuclei of an unstable atherosclerotic plaque show several hybridization signals (tetrasomy and polisomy). No numerical alterations of the chromosome 7 are observed in stable plaque. (Adapted from Matturri et al, 2001.)⁴¹

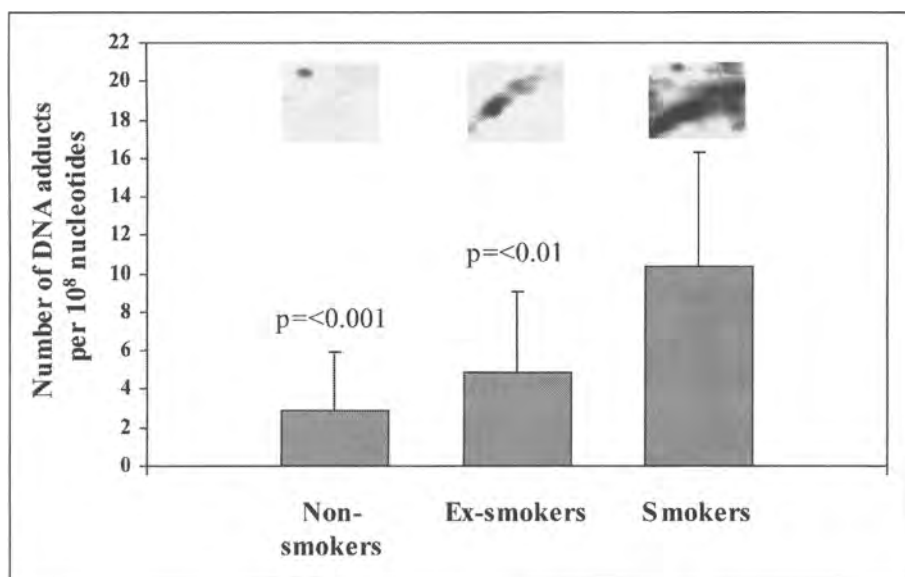


Figure 6. Levels and profiles of aromatic DNA adducts by the ^{32}P -postlabeling assay in right atrial appendages from nonsmoker, ex smoker, and smoker patients. (Adapted from Van Schooten et al, 1998.)⁴⁵

Increased levels of 8-hydroxy-2'-deoxyguanosine (8-OHdG), and repair-associated proteins, have also been observed in macrophage-derived foam cells in the atherosclerotic plaques of rabbits fed a cholesterol-rich diet.⁴⁶ The authors argue that during hypercholesterolemia-induced atherogenesis, ROS are formed resulting in oxidative damage to DNA. This DNA damage is followed by increased DNA repair activity so that initial damage is efficiently repaired. However, the cells may reach a point of no return, after which the DNA repair system can no longer cope with the extensive damage and apoptotic cell death becomes imminent, promoting both atherogenesis (via core formation) and plaque instability.⁴⁶ However, a significant decrease in 8-OHdG levels and DNA repair enzymes in atherosclerotic plaques of rabbit thoracic aorta has also been obtained upon lowering of dietary lipid, suggesting that an efficient removal of DNA adducts may contribute to stabilization of the plaque.⁴⁶ Furthermore, in human plaques, lesion-associated cells show higher 8-OHdG levels compared to similar cells not associated with plaque.⁴⁷ Similarly, increased oxidative damage to DNA molecule has been observed in leukocytes of patients with atherosclerosis compared with healthy subjects.⁴⁸⁻⁵⁰

We have demonstrated an elevation of DNA strand breaks as well as of oxidized pyrimidines and purines in patients with coronary artery disease by using the comet assay (Fig. 7).⁵⁰ Recently, it has also been reported that levels of DNA strand breaks in human lymphocytes are significantly higher in patients with acute coronary syndrome, than in patients with stable angina or healthy subjects.⁵¹ Furthermore, a significant increase of DNA strand breaks has also been observed in patients with acute myocardial infarction compared to patients with unstable angina (Table 1), suggesting that peripheral blood cell levels of DNA damage may be a factor of plaque instability.⁵¹

Finally, a recent study has also reported both loss of RNA integrity and increased 8-oxo-guanosine in advanced human atherosclerotic plaques compared with nonatherosclerotic mammary arteries.⁵² This observation supports a proposal that RNA damage may negatively affect a variety of distinct cellular processes in the plaque including cell survival and proliferation.⁵²

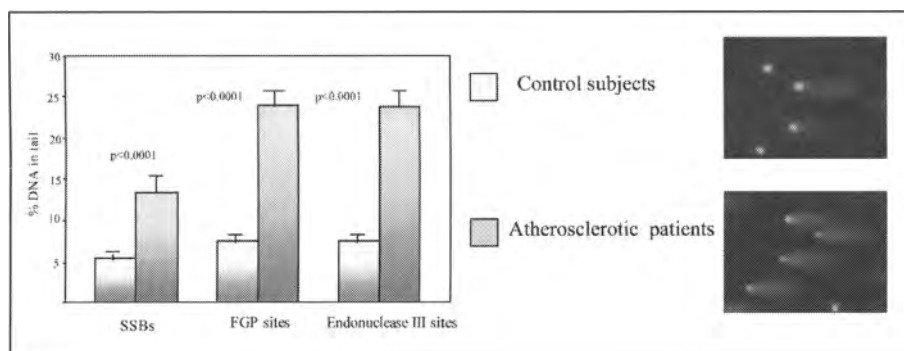


Figure 7. Oxidative DNA damage- as demonstrated by single strand breaks (SSBs), formamidopyrimidine glycosylase (FPG) sites, and endonuclease III (EIII) sites- in human lymphocytes of patients and controls by using comet assay. (Adapted from Botto et al, 2002.)⁵⁰

Table 1. DNA strand breaks in human lymphocytes of patients with coronary artery disease

Study Groups, n	DNA Strand Breaks, AU
Healthy subjects, 35	68 ± 34
Stable angina, 48	116 ± 37*
Acute coronary syndrome, 53	144 ± 52 [#]
Unstable angina, 29	131.8 ± 48.4
Acute myocardial infarction, 24	159.8 ± 53.0 [§]

Values are mean±SD. AU: arbitrary unit. * $p < 0.001$ versus healthy subjects. [#] $p < 0.001$ versus stable angina. [§] $p < 0.05$ versus unstable angina. (Adapted from Demirbag et al, 2005.)⁵¹

Somatic Mitochondrial DNA Mutations

At present, there is growing interest in the role of defects in mitochondrial bio-energetics as a cause of cardiomyopathy and vascular disease.⁵³⁻⁵⁶ Compared to nuclear DNA, mitochondrial DNA (mtDNA) is more vulnerable to oxidative DNA damage because of its proximity to sources of ROS generation in the mitochondria inner membrane, its lack of protective histones, and its limited capability for DNA repair.⁵⁷ Damage to mtDNA can lead to decreased energy production and increased generation of intracellular ROS.⁵⁸ Such aberrant mitochondria may contribute to a wide variety of pathologies, including cancer, diabetes and cardiovascular disease.⁵⁹ In cardiovascular disease, mitochondria are biologically important sources and targets for ROS.^{60,61} It is also reported that exposure of vascular cells to ROS in vitro results in preferential mtDNA damage and decreased mtDNA-encoded gene transcription in a dose-dependent manner.⁶² These findings suggest that the resultant decrease in cellular ATP levels and altered mitochondrial redox status may contribute to vascular disease, affecting both endothelial and vascular smooth muscle cell functions, including growth, signalling and death.⁶²

The importance of mitochondrial dysfunction in atherogenesis was also confirmed by Ballinger et al⁶³ using genetically altered mouse models (ApoE^{-/-} and ApoE^{-/-}/SOD2^{+/-}). In these models, mtDNA damage preceded the development of atherosclerosis in ApoE^{-/-} mice compared to age-matched control animals (Table 2). In addition, ApoE^{-/-} mice deficient in manganese SOD, a mitochondrial antioxidant enzyme, exhibited increased mtDNA damage and

Table 2. mtDNA lesions /10 kb in control and ApoE^{-/-} mice

	Control Mice mtDNA Lesions /10 kb	ApoE ^{-/-} Mice mtDNA Lesions /10 kb	p Value
10 weeks	0.001±0.198	0.582±0.123	0.018
34 weeks	0.453±0.162	1.325±0.257	0.007

Values are mean± SEM. (Adapted from Ballinger et al, 2002.)⁶³

atherosclerotic lesions compared with age-matched mice, suggesting a causative role for mitochondrial oxidative stress, specifically, in the development of atherosclerosis.⁶³ Furthermore, a murine model of myocardial infarction and remodelling has also clearly demonstrated an increased generation of ROS associated with mitochondrial damage in hearts after myocardial infarction.⁶⁴ This mitochondrial dysfunction was characterized by increased lipid peroxidation in the mitochondria, decreased mtDNA copy number and mtRNA transcripts, and reduced enzymatic activity of complexes I, III, and IV. In contrast, enzymatic activity of complex II and citrate synthase, encoded only by nuclear DNA, were unchanged.⁶⁴ In addition, increased mtDNA damage, as measured by increases in protective 8-oxo-dGTPase levels, has been observed in post-myocardial infarction mice hearts, suggesting that mtDNA damage is important in heart failure.⁶⁵

Recent findings have also pointed out that some cardiovascular risk factors (second-hand smoke and hypercholesterolemia) cause damage to mtDNA in cardiovascular tissue from mouse models.⁶⁶ It is known that mtDNA damage may include deletions, insertions, or a combination of the two.⁵³⁻⁵⁶

In particular, the most common deletion of mtDNA, and also the most often assayed, is a deletion that occurs between nucleotides 8,470 and 13,447 of the human mtDNA; it encompasses five tRNA genes and seven genes encoding sub-units of cytochrome c oxidase, complex I and ATPases.^{67,68} Although the incidence of mtDNA4977 is relatively low in normal hearts, the frequency of deletions in hearts from patients with coronary artery disease can be increased between 7- and 2,200-fold.⁶⁹ In our laboratory, we have also demonstrated a significantly higher incidence of mtDNA4977 deletion in peripheral blood cells of patients with coronary artery disease compared to healthy subjects.⁷⁰ Together, the above data indicate a strong correlation between mtDNA damage and the pathogenesis of atherosclerosis.

Conclusion

There is growing evidence to indicate that oxidative damage to DNA could affect cardiovascular cell function, and represents an important link between inflammatory and oxidative theories of atherosclerosis. However, our understanding of the mechanism by which oxidative DNA damage participates in the atherogenic process is still limited. Further investigations are necessary to clarify whether oxidative damage to DNA, observed in the atherosclerotic plaque, is implicated in early development of the disease, contributing to the genetic abnormalities of outgrowing SMCs, or is the consequence of other selective stimuli. Future studies should also focus on defining whether genetic instability may have a pathogenic role via a molecular mechanism which influences the transcription, replication or expression of vulnerable genes involved in atherosclerosis development. It is also vital to define how mutations and deletions in mtDNA affect signalling and cellular energy of both endothelial cells and vascular SMC, key components of atherogenesis. Clinical research must seek to define whether oxidative damage to DNA can be an additional prognostic predictor, evaluating a biological dimension presently ignored by current stratification risk. These studies may be particularly relevant for conceiving novel therapeutic approaches aimed at preventing or limiting the acute complications of atherosclerosis.

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CHAPTER 12

Oxidative DNA Damage and Carcinogenesis

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Abstract

In living cells, there is a steady formation of DNA lesions. A substantial number of these lesions are formed by endogenous factors such as reactive oxygen species (ROS) that damage DNA on a continuous basis. Therefore, it is likely that ROS are the most important human carcinogens.

An involvement of oxidative DNA damage, particularly 8-hydroxy-7,8-dihydroguanine (8-OHGua) in the origin and/or progression of cancer is reviewed. It is concluded that severe oxidative stress manifested as an altered level of 8-OHGua in cellular DNA as well as in urine of cancer patients may be a consequence of development of many types of cancer. Although at present it is impossible to answer directly the question concerning involvement of oxidative DNA damage in cancer etiology it is very likely that oxidative DNA base modifications may serve as a source of mutations that initiate carcinogenesis (i.e., they may be causal factors responsible for the process).

It should also be remembered that DNA damage, altered gene expression and mutations are required participants in the process of carcinogenesis. Although these events may be derived by different mechanisms a commonality is the involvement of oxidants in all these phenomena.

Introduction

Cancer is a disease of our genes. An increase in somatic mutations, which are the main cause of cancer development, has been documented in aged cells and tissues.¹ This accumulation of mutations presumably relates to a cumulative, lifetime exposure to endogenous and exogenous DNA damaging agents. Although many carcinogens can be found in food and drink generated during their processing (e.g., cooking process of meat) and as a result of the activities of the chemical industry, it is likely that the most important human carcinogens may be metabolites of atmospheric oxygen, reactive oxygen species (ROS). These ROS can be produced both during the biochemical utilization of oxygen (e.g., there are more than 60 enzymatic reactions that utilize O₂ as a substrate where ROS may be formed, such as oxidase-catalyzed reactions, autooxidation of reduced forms of NAD⁺/NADP⁺—dehydrogenase, xanthine oxidase, macrophage-catalyzed reactions and cytochrome P₄₅₀ proteins, to name a few)² and as a by-product of O₂ metabolism in mitochondria. ROS can damage different types of cellular molecules—proteins, lipids and nucleic acids. Free radical attack upon DNA generates a whole series of DNA damage products, among them modified DNA bases. Hydroxyl radical causes the formation of a large number of pyrimidine- and purine-derived lesions in DNA (reviewed in ref. 3). Some of these modified DNA bases have considerable potential to damage the integrity of the genome (reviewed in refs. 4,5). 8-Oxo-7,8-dihydroguanine (8-OHGua) is one of

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the most widely studied lesions. The presence of 8-OHGua residues in DNA leads to GC→TA transversions unless repaired prior to DNA replication.⁶ Therefore, the presence of 8-OHGua in cells may lead to point mutations.

Oxidative stress driven lipid peroxidation (LPO) is also a powerful source of endogenous DNA damaging agents, including 2,3-unsaturated aldehydes (enals) such as crotonaldehyde, acrolein and 4-trans-2-hydroxynonenal (HNE). These, relatively stable compounds may form unsubstituted and substituted exocyclic propano- type or etheno-type DNA adducts. The most extensively studied lesions, unsubstituted 1,*N*⁶-ethenoadenine (εA) and 3,*N*⁴-ethenocytosine (εC) reveal miscoding potential in mammalian cells leading to base substitutions and frame-shifts,⁷⁻⁹ as well as chromosomal aberrations and recombination.¹⁰ Propano- or etheno-type adducts bearing an alkyl side chain, e.g., HNE-DNA adducts, create a strong hindrance for DNA polymerases, but their presence in DNA also yields point mutations and recombination.¹¹

Many observations indicate a direct correlation between the formation of 8-OHGua and other oxidative stress derived DNA lesions and carcinogenesis *in vivo*.^{4,12} Oxygen-derived radicals are known to induce mutagenesis in hotspot codons of the human p53 and Ha-ras genes.^{13,14} Also, the lipid peroxidation product, HNE tends to react selectively with the guanine residue in p53 codon 249 sequence GAGG⁺C/A,¹⁵ which is the mutation hotspot in hepatocellular carcinoma, and other types of human cancer. Therefore, background oxidative damage to DNA may be critical to the appreciation of the importance of oxidative stress in the development of cancer.

The background level of 8-OHGua in cellular DNA represents a dynamic equilibrium between the rate of oxidative DNA damage and the rate of repair of the damage in specific tissues/cells. That repair enzymes which specifically recognize and remove 8-OHGua have evolved, is a clear indication of the biological significance of this lesion, but exactly how much 8-OHGua is present at the background level is a subject of controversy. Alternatively, an approach to assess oxidative DNA damage is the measurement of urinary excretion of 8-OHGua and its 2'-deoxyribonucleoside equivalent, 8-OHdG (8-oxo-7,8-dihydro-2'-deoxyguanosine).

This chapter reviews recent data concerning the involvement of oxidative DNA damage, particularly 8-OHGua, in the initiation and/or progression of cancer. In discussion of these issues, we will highlight recent advances from our laboratory, along with literature reports.

Accumulation of 8-OHGua in Cancer Patients

Cancer Tissues

We and others have demonstrated that elevated levels of typical free radical-induced DNA base modifications, including 8-OHGua, exist in some types of human cancers, in cancerous tissues when compared with the cancer-free surrounding tissue.¹⁶⁻¹⁹ It is not known whether these elevated levels of DNA lesions play a causative role in carcinogenesis or are merely the result of the disease. However, treatment of laboratory animals with carcinogenic agents causes a similar pattern of oxidative base modification in their target organs before tumor formation occurs.²⁰ As previously mentioned, several lesions which have been found in higher amounts in cancerous tissues,¹⁶⁻¹⁹ possess mutagenic properties. These data suggest an important role for oxidative DNA base damage in carcinogenesis.

Our recent investigations of benign tumors showed that oxidative DNA damage might be a causative factor in cancer development. A higher endogenous level of 8-OHGua in uterine myoma tissues was observed when compared to their respective tumor-free tissues.²¹ Uterine myomas are common gynaecological neoplastic lesions. They are monoclonal, benign tumors derived from a single mutated myometrial cell. One of the factors that may predispose malignant transformation is the greater size of the tumor.²² The positive correlation found in our work between the size of the tumor and the amount of 8-OHGua (Fig. 1) suggests that the higher level of 8-OHGua and possibly other base lesions in benign tumors may be a risk factor that may determine the transformation of benign to malignant tumors.²¹ Similarly, the increased levels of modified DNA bases may contribute to the genetic instability and metastatic potential of tumor cells in fully developed cancer.

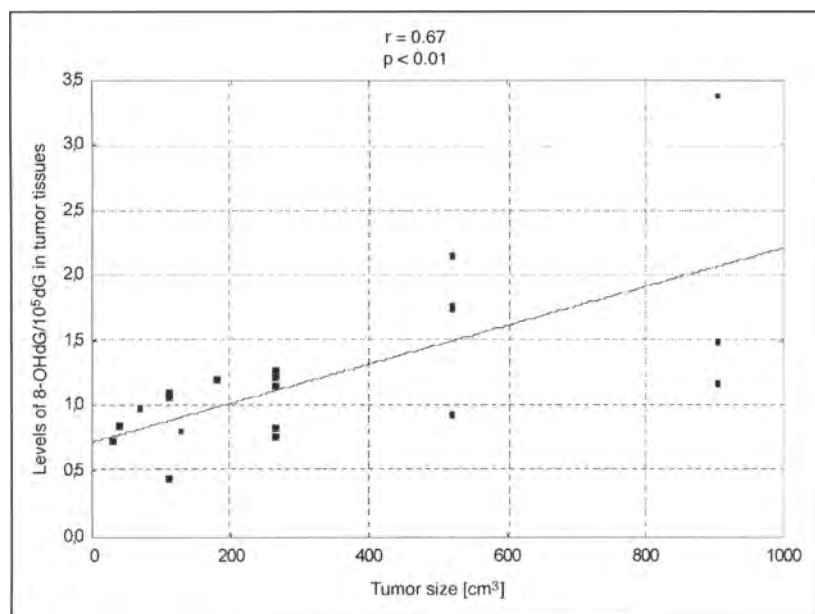


Figure 1. Correlation between 8-OHdG in myoma tumor tissues and tumor size. Reproduced from: Foksinski M et al; *Free Radic Biol Med* 2000; 29:597-601; ©2000 with permission from Elsevier.²¹

It has been estimated that most human cancers contain a large number of mutations. At least 11,000 individual DNA mutations exist in a single carcinoma cell of colorectal tumors.^{23,24} It is possible that some of these mutations may arise during the development of the disease and may contribute to the metastatic potential of tumor cells. Our results suggest that one potential source of this unusually large number of mutations may be damage to DNA by ROS. This explanation has also been suggested by Malins et al¹⁷ who showed that the metastatic potential of breast cancer tissues increased together with increases in the level of oxidatively modified DNA bases.

Urinary Excretion of 8-OHGua and 8-OHdG in Cancer Patients

Since the level of the modified nucleosides/bases in urine may be an indicator of oxidative insult on DNA and a general marker of oxidative stress, we investigated whether the amount of 8-OHGua and 8-OHdG excreted into urine was higher in cancer patients than in the control group. It was found that the amount of the modified base, but not the 2'-deoxynucleoside, excreted into urine was approximately 50% higher in cancer patients than in a corresponding control group.²⁵

The level of damaged DNA bases in urine can depend upon both the degree of oxidative DNA insult and activity of different repair mechanism(s). The higher level of 8-OHGua in the urine of cancer patients may be explained, at least partially, by the reported oxidative stress in cancer tissue.^{16,19,26,27} However, the amount of the modified base/2'-deoxynucleoside excreted into urine should represent the average rate of DNA damage in the whole body.^{28,29} Therefore, it is doubtful that the elevated level of the base product in cancerous cells alone could account for the observed 50% increase of 8-OHGua in urine. Our results suggest rather that oxidative stress, represented by the increased amount of 8-OHGua in urine, may be characteristic not only for the diseased tissue but also for some other tissues (or the whole organism) of cancer patients, i.e., a marker of generalised oxidative stress. The precise

mechanism(s) by which this oxidative stress arises is still unknown. However, some mechanisms may be suggested:

- i. It has been recently documented that cancer patients showed signs of extensive granulocyte activation with a release of ROS followed by a dramatic increase of 8-isoprostane, a biomarker of oxidative stress.³⁰
- ii. It has been shown that malignant cells can produce hydrogen peroxide at levels as large as those characteristic for stimulated polymorphonuclear leukocytes.³¹ Therefore, one of the reasons for the observed oxidative stress in advanced stages of cancer may be a release of the large number of cancer cells into the blood stream³² and their penetration into other tissues. Interestingly, it has been demonstrated that exposure to activated leukocytes causes oxidative DNA base modifications (among them 8-OHGua) in target cells.³³
- iii. Still another reason for the observed phenomenon could be that some tumors may stimulate the defense systems of the body so that they react against the tumor to produce cytokines.³⁴ Some of the cytokines can be responsible for ROS production.^{35,36} For example, it has been shown that an elevated plasma level of TNF is responsible for increased oxidative DNA damage of CD 34⁺ cells.³⁷

It is also possible that a pro-oxidant environment is characteristic of advanced stages of cancer and that generalised oxidative stress is a result, rather than a cause, of the disease development.

Oxidative DNA Damage Repair, Mutagenesis and Carcinogenesis

To combat the deleterious biological effects of the presence of 8-OHdG, cells have developed specific mechanisms to remove this lesion from cellular DNA. From bacteria to mammals three enzymes form "GO" systems that are involved in protecting cellular DNA against the mutagenic effects of 8-OHdG. The first level of this protection is MTH, a protein with pyrophosphohydrolase (8-OHdGTPase) activity. This enzyme is responsible for hydrolysis of 8-OHdGTP to 8-OHdGMP, thereby eliminating it from the nucleotide pool, and preventing its incorporation into DNA by DNA polymerases. The second level of defense is/are specific DNA-glycosylase(s) that initiate the base excision repair (BER) pathway. The major 8-OHGua glycosylase in *E. coli* is the Fpg protein (MutM), and a more recently discovered activity of endonuclease VIII (Nei), originally discovered as a damaged pyrimidine-specific DNA glycosylase.³⁸ MutM preferentially excises 8-OHGua when paired with C, T and G, the Nei glycosylase is more specific for an 8-OHGua A mispair.

In humans two 8-OHGua glycosylases have been identified, OGG1 (8-oxo-guanine glycosylase) a functional but nonstructural analog of the Fpg protein specific to 8-OHGua C mispair³⁹ and OGG2 specific to 8-OHGua mispaired with G or A.⁴⁰ It was postulated that the OGG1 type glycosylase may be a housekeeping enzyme which removes 8-OHGua from DNA of nondividing cells whereas OGG2 may be more specific for repair of 8-OHGua in nascent or transcriptionally active DNA.⁴¹ Two Nei-like human glycosylases, NEIL1 and NEIL2, specific to oxidized pyrimidines, but also excising 8-OHGua, have been characterized. These glycosylases exhibit an unusual ability to excise oxidized bases both from dsDNA or from ssDNA.^{42,43} However, the major 8-OHGua DNA glycosylase in human cells is probably OGG1. Finally, the third level of repair is realized by MYH proteins that remove adenine mispaired with 8-OHGua.

There are numerous experimental data which suggest that decreased activity of the enzymes comprising the "GO" system may be linked with cancer development. It has recently been demonstrated that many types of DNA repair pathways are reduced in cancer patients and/or in tumors.⁴⁴ The precise mechanism(s) of this reduced repair capacity is still unknown. However some mechanisms may be suggested:

- i. It has recently been documented that tumor lung tissue of lung cancer patients showed loss of heterozygosity at the hOGG1 gene locus.^{45,46} The patients exhibiting loss of heterozygosity contained higher level of 8-OHdG adducts.⁶

- ii. It has been suggested that polymorphisms in DNA repair genes may be associated with differences in the repair efficiency of DNA damage.⁴⁷ Some studies suggest involvement of a hOGG1 polymorphism in lung cancer development.⁴⁸ Few polymorphic changes of OGG1 gene have been described, with the most common Ser326Cys, which is characterized by a lower enzyme activity.⁴⁹ It was suggested that the presence of two hOGG1 326Cys alleles confers a 2-fold increased risk of lung cancer,^{48,50} and also an elevated risk of prostate cancer and nasopharyngeal carcinoma.⁵¹ Inherited variants of hMYH at conserved amino acids are associated with somatic GC→TA transversions in colorectal tumors.⁵²

In our study,⁵³ for the first time, in the case of lung cancer patients and matched control groups, we have measured all the parameters, which may represent oxidative DNA base damage. Besides urinary excretion of the modified base and deoxynucleoside, the level of 8-OHdG in leukocyte DNA and leukocyte 8-OHGua repair capacity by BER enzymes was also analyzed. The level of 8-OHdG in DNA isolated from leukocytes of cancer patients was significantly higher than in DNA isolated from the two control groups of smokers and nonsmokers. Since oxidative DNA insult represented by urinary excretion of oxidative DNA lesions was similar in cancer patients and the control group with similar smoking status, it appears likely that a higher rate of generation of oxidative damage in cellular DNA of lung cancer patients is a result of deficiencies in DNA repair mechanism(s) (most likely BER) in this group.

This suggestion was confirmed with the measurement of 8-OHGua repair activity in leukocytes of the both smoker groups. In the smoking cancer patients this activity was significantly lower than in healthy smokers. Similarly Paz-Elizur and coworkers⁵⁴ observed lower 8-OHGua excising activity in blood leukocytes of lung cancer patients than in healthy controls, pointing to the possible role of 8-OHGua repair as a risk factor for developing lung cancer. It was also shown that *OGG1* knockout mice are predisposed to develop lung carcinoma and 8-OHGua was found to accumulate in their DNA.⁵⁵

In our recent study⁵⁶ we found that 8-OHGua level in human DNA is determined not only by its excision rate, but also by the frequency of its incorporation from the nucleotide pool into DNA by DNA polymerases, and the latter may be the most important contributor. We have studied 8-OHGua level in DNA, OGG1 repair activity, and hMTH1 activity in tumors and surrounding lung tissue, without histological changes (normal lung) of lung cancer patients. We found that 8-OHGua level was lower in tumor than in normal lung tissue, OGG1 activity was also lower in tumor, but hMTH1 activity was higher in tumor than in normal lung (Fig. 2). The activity of hMTH1 was three orders of magnitude higher than that of hOGG1 (picomoles of 8-OHGua versus nanomoles of 8-OHdGTP hydrolysed per hour per milligram of protein). This tremendous difference can be attributed mostly to differences in the turnover of these enzymes, since the expression of hMTH1 and OGG1 mRNAs is similar.⁵⁷ The k_{cat} values are 211 min^{-1} and 0.1 min^{-1} for hMTH1⁵⁶ and hOGG1,⁵⁸ respectively. The role of hMTH1 protein is further highlighted by the observation that overexpression of hMTH1 protein in mismatch deficient cell lines decreased the mutation rates to normal and reduced microsatellite instability which was accompanied by a reduction of the 8-OHdG level in DNA.⁵⁹ Also, expression levels of hMTH1 mRNA were inversely proportional to the levels of 8-OHdG in DNA in 11 human lung cancer cell lines and SV-transformed nontumorigenic human bronchial epithelial cells.⁶⁰ The higher activity of 8-OHdGTPase also coincided with lower background levels of 8-OHdG in DNA of foetal compared with maternal mouse organs.⁶¹

Surprisingly we observed decreased 8-OHGua excision activity in tumor lung tissue in comparison with nonaffected surrounding areas. The mechanism of this decrease is not known, but probably is not related to mutations in hOGG1 gene in tumors, since they have been found in only 4% of human kidney cancers, and were also sporadic in lung cancers.⁶² Some studies have shown frequent allelic loss of chromosome fragments at the position of the *hOGG1* gene in cancer tissue. Accordingly, a decrease of *hOGG1* expression was observed, e.g., in head

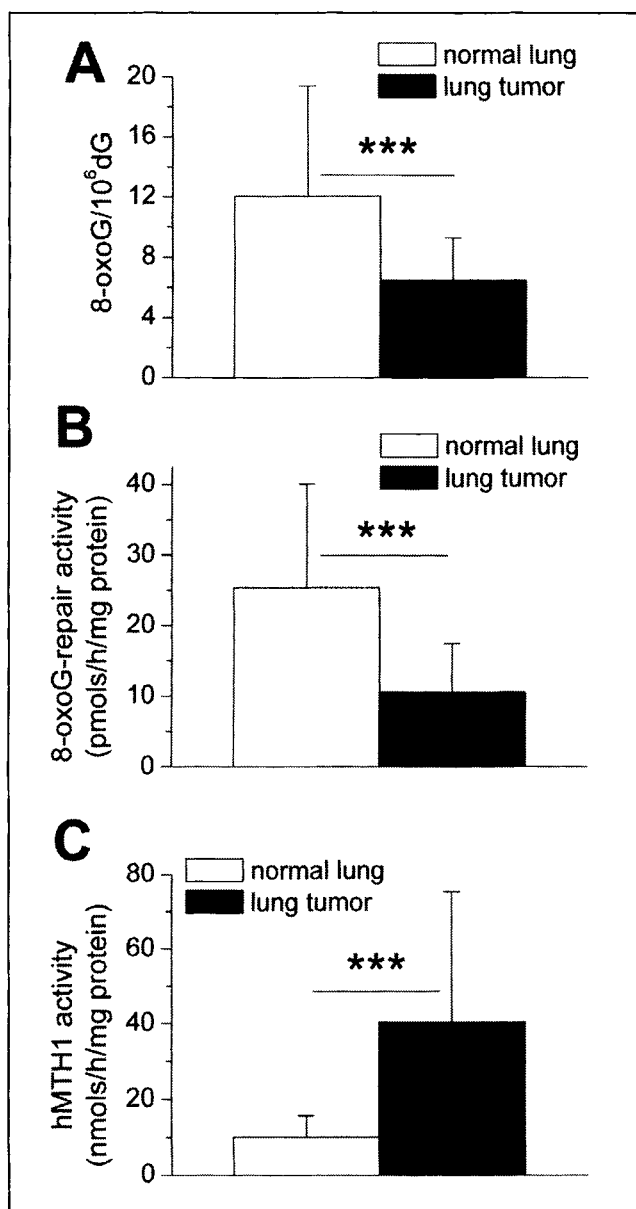


Figure 2. 8-OHGuA level in DNA (A), hOGG1 (B), and hMTH1 (C) activities in normal lung and tumor tissues of patients with nonsmall-cell lung cancer. *** $p < 0.001$. Reproduced from: Speina E et al; J Natl Cancer Inst 2005; 97:384-395; ©2005 with permission from Oxford University Press.⁵⁶

and neck squamous cancer cases.⁶³ However, loss of heterozygosity in the *hOGG1* locus may vary between cancer types. No differences in OGG1 expression were observed between tumour and nonaffected surroundings in human lung and kidney cancers.⁶² In model systems OGG1

activity is stimulated by at least three proteins, AP-endonuclease, hAP1 and NEIL1 glycosylase, which increase OGG1 turnover on damaged DNA,^{58,65} as well as XRCC1, which accelerates Schiff base formation between OGG1 and 8-OHGua, as well as orchestrating all steps of 8-OHGua repair.⁶⁴ One cannot exclude deregulation in tumour tissue of OGG1 cooperation with downstream partners of the BER pathway. The decrease in OGG1 activity may also be tumour-specific, driven by loss of OGG1 activators in tumour tissue. One such gene may be a tumour suppressor protein—tuberin. In tuberin deficient Eker rats, which spontaneously develop renal cancers, OGG1 expression and activity were reduced 3-fold.⁶⁶

It is also possible that increased oxidative stress in tumor tissue may directly inactivate some repair enzymes. Exogenous nitric oxide and peroxynitrite have been shown to inhibit hOGG1,⁶⁷ DNA ligase,⁶⁸ formamidopyrimidine-DNA-glycosylase⁶⁹ and O⁶-alkylguanine-DNA-alkyltransferase⁷⁰ by direct nitrosylation. However, there are conflicting data concerning the production of nitric oxide in human lung adenocarcinoma. Fujimoto et al⁷¹ reported higher nitric oxide synthase isoform activities in adenocarcinoma compared to other types of lung cancer and in normal lung, while Ambs et al⁷² did not find any upregulation of the synthase isoforms during nonsmall cell lung carcinoma (NSCLC) progression.

We also assessed the role of oxidative stress-driven LPO in the pathogenesis of lung cancer.⁷³ We measured the levels of ϵ A and ϵ C in the DNA by immunoaffinity/³²P postlabeling, as well as the repair capacity for ϵ A and ϵ C (by the 'nicking' assay) in normal and tumor lung tissues, as well as in blood leukocytes of lung cancer patients and healthy volunteers, matched with cancer patients for age, sex and smoking habit.

In humans ϵ A is eliminated from DNA by alkylpurine-DNA-N-glycosylase (ANPG),⁷⁴ and ϵ C by mismatch specific thymine-DNA-glycosylase (TDG).⁷⁵ Both enzymes are monofunctional DNA-glycosylases and require AP-endonuclease to incise DNA at the site of the removed base. Moreover, the activity of TDG is stimulated several fold by human AP-endonuclease, which increases the turnover of the enzyme on damaged DNA.⁷⁶ Thus, tissue repair capacity for ϵ C and ϵ A may depend on the availability of DNA-glycosylases and AP-endonuclease.

In contrast to 8-OHGua, no difference in ϵ A and ϵ C level between tumor and nonaffected lung tissues was recorded. Repair activities for ϵ A and ϵ C were significantly higher in tumor than in normal lung tissue. No significant differences in ϵ A and ϵ C-repair activities were associated with age, sex and smoking habit. However, significant difference in repair capacity was observed between two histological types of NSCLC, squamous cell carcinoma (SCC), which is related to the sensitivity to tobacco smoke components, and adenocarcinoma (AD), linked to chronic infections and healing of scars. In individuals suffering from lung AD, ϵ A and ϵ C-repair activities in normal lung and blood leukocytes were significantly lower than in SCC patients. Differences have also been found between ϵ A and ϵ C repair activities of cancer patients and healthy volunteers. Repair capacity for ϵ A was significantly lower in blood leukocytes of lung cancer patients than in leukocytes of healthy volunteers. This difference was even bigger between healthy volunteers and patients developing inflammation-related AD. In contrast repair activities for ϵ C were the same in leukocytes of healthy controls, all lung cancer patients and SCC patients. However, individuals with lung AD revealed significantly lower ϵ C-repair activity.

These results suggest that oxidative stress-mediated LPO might contribute to induction and/or progression of lung cancer. Decreased activity of BER for ϵ A and ϵ C is associated particularly with inflammation-related lung AD (Fig. 3). Lung AD has also been linked to defective repair of 8-OHGua. Polymorphism in the OGG1 gene⁷⁷ and downregulation of hMTH1 expression have been demonstrated in AD in comparison to SCC types of lung cancer.⁵⁷

Thus the development of histological types of NSCLC may be related to different causative factors, and among them is the deficiency of different repair pathways of oxidative stress-induced DNA damage.

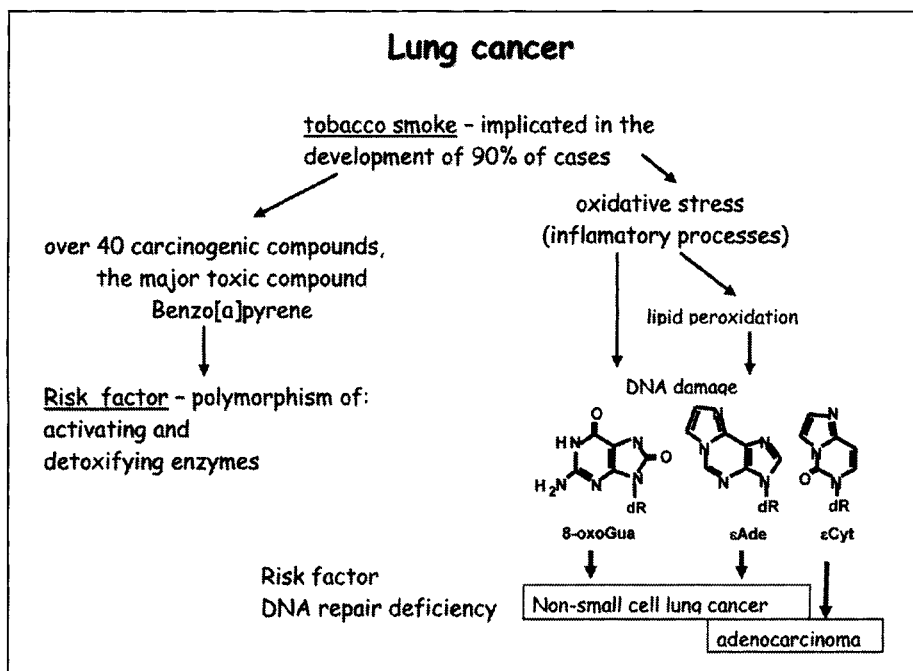


Figure 3. Involvement of oxidative DNA damage and its repair in lung cancer development.

Aging, Cancer and Oxidative DNA Damage

There is a dramatic age-dependent escalation in cancer risk and sequential accumulation of somatic mutations over a life time may be responsible for this phenomenon. An increase in somatic mutations was documented in aged cells in both humans and mice. The age-dependent accumulation of mutation may be directly linked to cumulative exposure to endogenous ROS.

DNA damage is considered of prime importance in aging.^{53,78} Free radical induced oxidative DNA damage is believed to be a major cause of aging-related DNA damage.^{79,80} Since oxidatively modified DNA bases have mutagenic potential⁸¹ their accumulation with time might be a major cause of the physiological changes associated with aging. There are multiple repair pathways to excise oxidative base modifications and prevent their incorporation into DNA.⁸² Following excision from DNA, the oxidatively induced lesions are released into the blood stream and consequently into the urine where their measurement has been acknowledged to be, at the very least, reflective of overall oxidative stress.²⁹

In our study we decided to analyze urinary excretion of possible repair products of oxidative DNA damage: 8-OHGua, 8-OHdG and 5-(hydroxymethyl)uracil (5-HMUr), in mammalian species that substantially differ in metabolic rate and longevity, namely mice, rats, rabbits, dogs, pigs and humans.⁸³ We found highly significant, positive correlations between specific metabolic rates of the studied animals and their excretion rates for all the analyzed modifications. It has also been found that 8-OHGua significantly correlates negatively with maximum life span (Fig. 4).

Our results demonstrated that ROS continually damage DNA and that this damage in vivo, in normal conditions is lower in long-lived species than in short-lived species. Incomplete repair of such damage would lead to its accumulation over time and eventually result in age-related pathologies such as cancer.

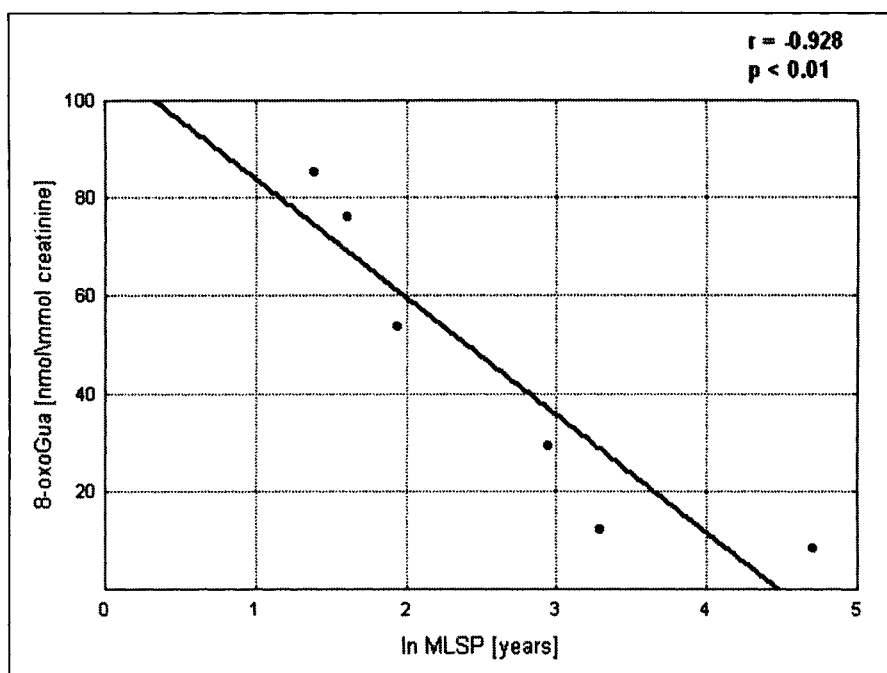


Figure 4. Relationship between the urinary excretion rates of 8-OHGua and natural logarithm from maximum life span of six different mammalian species. Reproduced from: Foksinski M et al; *Free Radical Biol Med* 2004; 37:1449-1454; ©2004 with permission from Elsevier.⁸³

Expression of the urinary excretion rates in nmol/kg/24 h enables measurement of the number of repaired lesions per day per cell.⁸⁴ Interestingly, urinary levels of all measured modifications, found in our study accounted for about 28,200 repaired events per average cell of the mouse per day and fits well with the estimation of Hamilton and coworkers⁸⁵ who calculated that the DNA of the liver cell in mouse acquires about 47,000 8-OHGua lesions in a 24 hour period (taking into consideration that the liver is a high metabolic rate organ and that our values are an average for the whole organism). In contrast, the number of all lesions analyzed in our work, in humans accounts for about 2,800 repair events in the average cell per day. It is therefore possible that high metabolic rate in mouse (or other short lived animal) may be responsible for severe everyday oxidative DNA insult that may be accumulated faster than in long-lived species. To the best of our knowledge these values are the first estimates based on the analyses of urinary excretion of several lesions. It is also noteworthy that the values estimated by us are in good agreement with those calculated for total oxidative DNA lesions.⁸⁴

Role of Inflammation in ROS Generation and Oxidative DNA Damage—Association with Cancer Development

It has been estimated that chronic inflammation may be involved in the development of about one quarter of all cancer cases worldwide.^{86,87} The inflammatory response can lead to the recruitment of activated leukocytes which in turn is directly linked to the “respiratory burst”—an increased uptake of oxygen that causes release of high quantities of ROS such as superoxide and hydrogen peroxide. Hydrogen peroxide can cross cellular and nuclear membranes and reach the nucleus to cause site specific DNA damage by producing $\cdot\text{OH}$ radical in reaction with DNA bound metal ions. Direct proof of this suggestion comes from the work of

Dizdaroglu et al³³ who demonstrated that exposure to activated leukocytes caused DNA base modifications in human cells typical of those induced by hydroxyl radical attack.

In other work it has been shown that exposure of DNA to either neutrophils or eosinophils activated in media containing metal ion chelators resulted in 8-OHGuA formation through a pathway that was blocked by peroxidase inhibitors, hypohalous acid scavengers, and catalytically active catalase and SOD.⁸⁸ Activated leukocytes oxidatively damage DNA, RNA and the nucleotide pool through halide-dependent formation of hydroxyl radical.⁸⁸

Ulcerative colitis (UC) is an example of chronic inflammatory disease associated with increased risk of colon cancer development. The colonic mucosa of patients with UC show symptoms of continuous inflammation which is associated with increased ROS production and decreased antioxidant defense.⁵ Oxidative DNA damage in the mucosa of patients with UC accumulated with the duration of the disease reaching maximal values in dysplastic lesions.⁸⁹

Various types of infection can initiate an inflammatory response and cancer development. *Helicobacter pylori* infection is the major etiologic factor responsible for gastric carcinogenesis. There is numerous experimental evidence suggesting that the chronic inflammatory reaction caused by the bacterial infection may be directly involved in the production of ROS, which in turn may lead to oxidative DNA damage and consequently to carcinogenesis. Increased oxidative DNA damage was detected during early stages of *Helicobacter pylori* infection.⁹⁰

Chronic infection with hepatitis B or C virus (HBV, HCV) may lead to hepatocellular carcinoma (HCC). It was demonstrated that in transgenic mice model of HBV there was accumulation of 8-OHdG in liver tissue early in life and the damage increased with advancing disease.⁹¹

The core protein of HCV has been shown to induce hepatocellular carcinoma in transgenic mice and this protein may play a significant role in the development of HCC in chronic HCV infection.⁹² One of the possibilities that can explain this phenomenon is the induction of oxidative stress via the core protein. It was found that as a consequence of the core protein expression there was an age-dependent increase in oxidative stress in the livers of the transgenic mice. This oxidative stress was independent of inflammation and may be directly involved in the development of HCC under HCV infection. Interestingly, it was demonstrated that HCV core protein may not only induce ROS production in hepatocytes but can accelerate ROS production when stimulated by such HCC associated factors as alcohol or inflammation.⁹³

Persistent infection with either HCV or HBV leads to higher levels of promutagenic 8-OHdG in human liver.⁹⁴ It is worth noting that the infection is associated with increased cellular proliferation, which may lead to fixation of promutagenic changes into mutations. Therefore, this increase of the content of oxidative DNA damage may be directly linked with HCC development.

Conclusions

It is clear that significant gaps exist in the current state of knowledge related to the possible causative link between oxidative DNA damage and cancer development. However, it is evident that oxidants may act at several stages in malignant transformation since there is a close link between ROS formation and oxidative DNA damage.⁹¹

There is increasing evidence that most human cancers contain large numbers of mutations.⁸⁹ This in turn suggests that they are generated continuously during tumor progression. Endogenous cellular processes (oxidative phosphorylation, peroxisomal fatty acid metabolism, cytochrome P-450 reactions or "respiratory burst" of phagocytic cells) are obvious sources of ROS that may be responsible for oxidative DNA base modifications and may serve as a source of mutations that initiate carcinogenesis. Since severe oxidative stress is also characteristic for advance stages of cancer development, these modifications may also serve as an efficient source of mutations during tumor progression. In order to contribute to mutations, oxidative DNA damage would need to occur at a sufficiently high frequency to exceed the capacity of the cell for DNA repair. In this context it is noteworthy that in our urinary excretion study, the average

8-OHGua and 8-OHG excretion in urine of healthy subjects was 2.5 nmol per kg per day, corresponding to about 2000 oxidative modifications of guanine per cell per day.

To sum up: in light of the presented data, it is likely that severe oxidative stress is a consequence of development of many types of cancer. However, at present it is impossible to directly answer the question concerning the involvement of oxidative stress in cancer origin since full development of the disease in response to carcinogen exposure takes 20–40 years. Therefore, it is very difficult to prove directly that DNA oxidation is responsible for carcinogenic processes, as the lesions are present in tumors many generations later. Nevertheless, it should be remembered that DNA damage, altered gene expression and mutations are required participants in the process of carcinogenesis. Although these events may be derived by different mechanisms, a common feature is the involvement of oxidants in all these phenomena.

Acknowledgement

R. Olinski is supported by a Foundation for Polish Science fellowship. The authors also acknowledge financial support from the EU NoE "ECNIS" grant # 513943 and from the State Committee for Scientific Research grants PBZ-KBN-091/P05/2003/55 and 3 PO5A 019 25.

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CHAPTER 13

The Physiological and Pathological Roles of Oxidative Damage to DNA in Relation to Life Stage

Alberto Izzotti*

Abstract

Oxidative damage to DNA (ODD) is a common phenomenon occurring during all life stages in all aerobic organisms. To evaluate the biological significance of ODD, we monitored 8-hydroxy-2'-deoxyguanosine, lipophilic bulky DNA-adduct formation, and the expression of oxidative-stress related genes in a variety of animal and human studies.

In mouse foetal liver, the low basal level of ODD was increased following transplacental exposure to cigarette smoke. The foetus counteracted ODD by increasing the expression of genes inhibiting cell replication and triggering apoptosis. Accordingly, smoke-induced ODD in the foetus results in growth retardation. During the foetus-newborn transition, the acquisition of independent respiratory function triggers the expression of genes involved in the detoxification of reactive oxygen species and removal of oxidized proteins, as demonstrated in mouse lung. The most abundant ODD accumulation during lifetime, contributing to ageing, was detected in mouse heart and brain.

In humans, ODD is consistently detectable in the aorta of atherosclerotic patients, being 4-fold higher in the inner than in the medium layer.

To substantiate the hypothesis that ODD is related to various chronic-degenerative diseases, we analysed 8-hydroxy-2'-deoxyguanosine in the trabecular meshwork, the epithelium regulating the intra-ocular pressure, of patients affected by primary open angle glaucoma, the main cause of irreversible blindness worldwide. There was a significant ($p < 0.001$), 3.4-fold, increase in ODD in glaucoma patients as compared to unaffected controls. This situation leads to an increase of intra-ocular pressure resulting in optic nerve alterations and visual field defects.

Altogether, these data support the view that ODD is involved in a variety of physiological processes (e.g., birth and ageing) and pathological conditions (e.g., intrauterine growth retardation, atherosclerosis and glaucoma).

Introduction

Oxidative damage to DNA (ODD) is a common phenomenon occurring in all aerobic organisms. The total ODD level is usually very high as compared to the other types of DNA damage. Typically, in organisms devoid of any exposure to environmental genotoxic agents, the burden of oxidative DNA alteration, measured in terms of 8-hydroxy-2'-deoxyguanosine (8-OHdG), falls

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in the order of 1.8-OHdG molecule every 10^5 - 10^6 normal nucleotides, while by comparison the total burden of molecular DNA alteration, measured in terms of lipophilic bulky DNA adduct, is in the order of 1 adduct every 10^8 - 10^9 normal nucleotides. This makes oxidative alterations of nucleotides the most common DNA lesions occurring under physiological conditions in organisms in the absence of exposure to exogenous genotoxic stimuli. A general trend towards a progressive increase in ODD accumulation from the early to the late life stages exists (Fig. 1). However, physiological variations related to organism development may remarkably influence the ODD rate. These situations do not possess a pathological importance per se, but simply represent the physiological consequence of aerobic life. This physiological trend may be altered by a variety of stress conditions abnormally increasing ODD outside its physiological amount. Under these conditions, exceedingly high ODD may represent a risk factor for the appearance of a variety of pathological conditions at all life stages (Fig. 1, dotted curves). The pathological consequences of ODD accumulation may be different depending on a variety of factors including the stage of life at which they are occurring, the affected organ, and the inducing cause.

Both endogenous and exogenous sources lead to ODD, whose reciprocal contribution is different during the various life stages. Whenever their combination results in an exceedingly high accumulation of oxidative damage, and also because of the existence of contributing factors such as a decreased ODD removal, a pathological situation may occur. Because of this, ODD-related degenerative diseases encompass exogenous and endogenous risk factors whose interplay determines the probability of developing the disease.

Based on these premises, it can be stated that ODD may possess a physiological or pathological role depending on its amount as related to a specific life stage. This chapter illustrates a variety of animal experimental models and human clinical studies supporting the view that ODD may be related to a variety of physiological or pathological situations arising during an organism's lifetime.

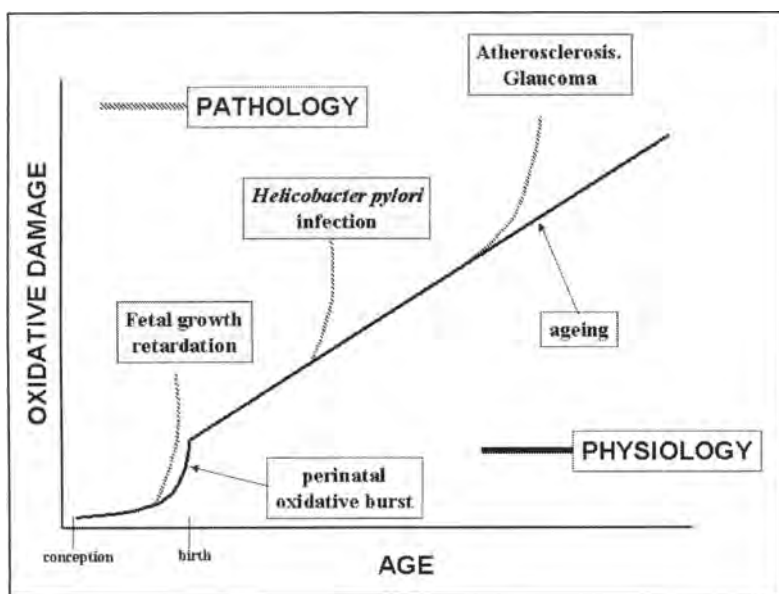


Figure 1. Life-time accumulation of oxidative DNA damage (ODD), as summarized from results of the experimental and clinical studies reported in this chapter. The basal line indicates the physiological ODD level occurring at different life stages. Dotted curves indicate examples, as discussed in the text, of pathological consequences of exceedingly high ODD accumulation as occurring at different life stages.

Early Life Stages: Intrauterine Life

Physiological Situation

Several studies have been initiated to analyse ODD occurring during intrauterine life as compared to extrauterine life. Results indicate that the level of ODD is remarkably low during intrauterine life. We monitored 8-OHdG in various organs of mice, devoid of any exposures to genotoxic agents, during their lifetime. Tested animals included foetuses, newborn, and 1 and 2 year-old mice, with liver, brain and heart analysed. Results are shown (Fig. 2), which documents the data pooled from both published studies¹⁻³ and unpublished data (Izzotti et al unpublished data). Up until 1 year old, the liver contains higher levels, although below the statistical significance threshold, of 8-OHdG, a finding likely to be related to the high metabolic rate of this organ. However, a dramatic and significant increase in ODD was detected during the late life stages in brain (2.0-fold in 2- compared to 1-year old animals) and heart (2.96-fold increase), while such an increase (1.45-fold) was not statistically significant in liver (Fig. 2). In all tested organs, the lowest ODD level was detected during early life stages and in particular during intrauterine life. Similar findings were also obtained by analysis in foetal and adult (3-months old) mice endogenous lipophilic bulky DNA-adducts derived from oxidation as detected by ³²P-postlabelling. These adducts have been identified as a variety of nucleotide modifications resulting from ODD, including adduction of reactive lipid peroxidation products to DNA.⁴ Pooling data from various studies^{2,3} and from unpublished data (Izzotti et al), their amounts were 7.32-fold, 8.87-fold, and 5.62-fold lower in foetus than in adult in liver, heart, and lung respectively (Fig. 3). An interpretation of these findings could be that the foetus is shielded against oxidative stress by effective antioxidant defences of the mother, defences which are fully developed in the adult organism and also powerfully induced by the endocrine situation characterising the pregnancy period. Therefore, the low ODD level detected in various foetal organs is related to the fact that foetus, under physiological conditions, is not directly exposed to exogenous oxidising sources and is actively protected against endogenous oxidants by the maternal antioxidant machinery.

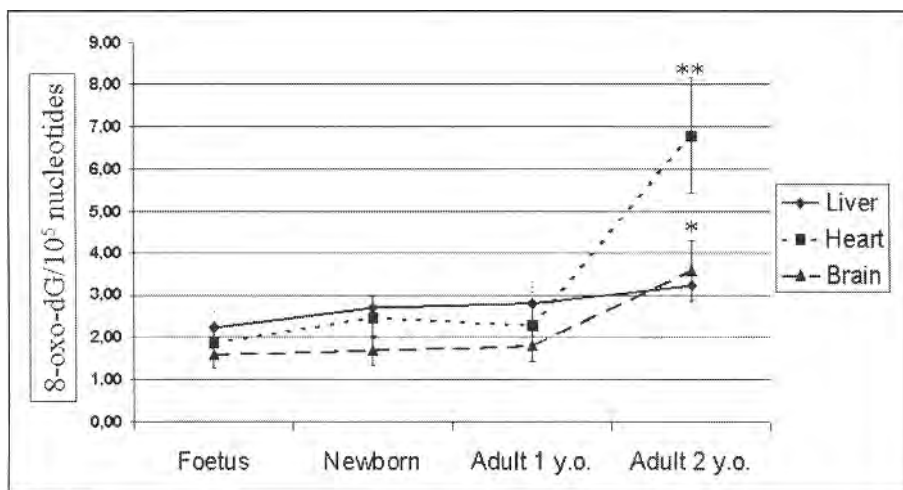


Figure 2. Levels of 8-hydroxy-2'-deoxyguanosine as detected in various mouse organs at different ages. Significant (* $P < 0.05$; ** $P < 0.01$) increases were observed in heart and brain of 2-years old as compared to 1-year old mice.

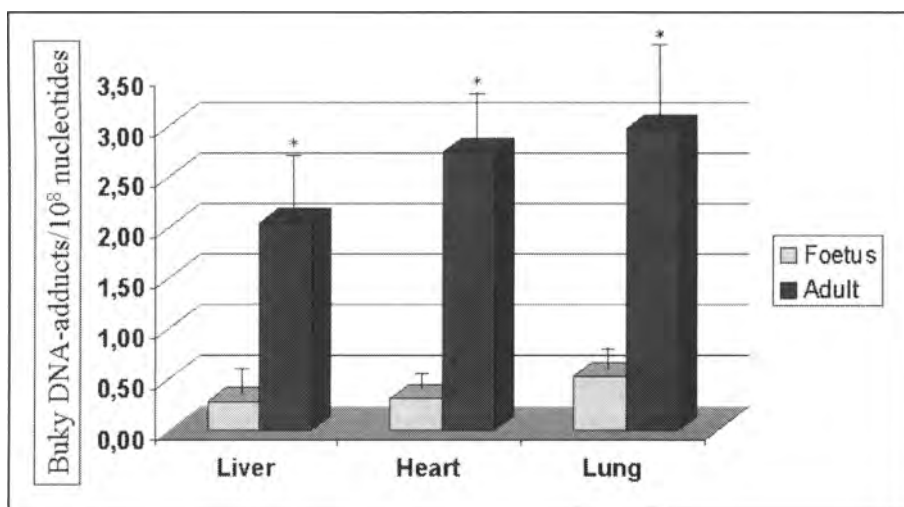


Figure 3. Oxidative DNA-damage (lipophilic bulky endogenous DNA adducts) as detected in various organs of foetus and adult mice. Adduct levels were significantly (* $P < 0.05$) higher in adults than in foetus in all organs tested.

Pathological Conditions: Intrauterine Growth Retardation

Because of the considerations noted above, the foetus does not possess active enzymic antioxidant defences, becoming fully developed later in life. An analysis of gene expression, as performed by cDNA array, in liver³ and lung (Izzotti et al unpublished data) of mouse foetuses indicates that the vast majority of 746 tested genes, involved in stress responses, are still silent. Inactive genes include activities encoding for antioxidant metabolic pathways (e.g., superoxide dismutase and catalase), removal of oxidative-damaged proteins (e.g., heat shock proteins, T-complexes, crystallins), and repair of oxidative lesions to nucleotides (e.g., 8-oxo-dGTPase and OGG1). These data provide evidence that DNA repair functions in foetuses are dramatically low, as also demonstrated by other authors in rat⁵ and humans.⁶

This physiological situation causes a differential susceptibility to the formation and persistence of ODD in foetal as compared to adult organisms. Following exposure to oxidative stress, the defensive mechanisms of the foetus cannot fully activate metabolic-detoxification and DNA-repair pathways. Accordingly, the main defensive mechanisms activated involve biological functions already functional during intrauterine life, i.e., cell-cycle regulation and removal of ODD-bearing cells by apoptosis. This hypothesis has been supported in mouse foetuses exposed transplacentally to cigarette smoke.³ Such exposure results in a dramatic increase in ODD in foetal liver, as demonstrated by the significant increases of 8-OHdG (4.6-fold) and lipophilic bulky DNA-adducts (6.3-fold), in smoke as compared to sham-exposed animals. Under this experimental situation, ODD is the consequence of the direct oxidising effect of cigarette smoke components.⁷ Additionally, inflammation is dramatically induced in liver and lung of mouse foetuses transplacentally exposed to cigarette smoke,⁸ contributing further to ODD formation. Finally, ODD in smoke exposed organisms also derives from damages affecting the major endogenous sources of reactive oxygen species, i.e., mitochondria, whose DNA was demonstrated to be very sensitive to smoke-induced DNA damage.⁹

The foetus reacts to ODD by triggering the expression of a variety of genes, mainly devoted to decreasing cell-replication rate and to increasing apoptosis, as demonstrated by analysing the expression of 746 stress response genes by cDNA array.³ Cell-cycle regulation and apoptotic processes are already active in the foetus because of the morphogenic processes involved in the

developing organism. These defensive mechanisms are quite effective, as genotoxic damage, evaluated in terms of micronuclei formation, following a sudden initial increase reaches a plateau and decreases during the latter stages of intrauterine life (Balansky et al personal communication). The high efficiency of these defensive mechanisms is likely to explain the lack of a clear association between the risk of developing cancer and the intrauterine exposure to cigarette smoke, as reported by epidemiological studies.¹⁰ The only cancers associated with transplacental smoke exposure are leukemias and lymphomas, probably related to lymphocyte damage in the foetal haematopoietic liver.⁸ Furthermore, genes encoding for leukocyte activation, such as granulocyte-macrophage colony stimulating factors, have been demonstrated to be significantly upregulated in the liver of mouse foetuses transplacentally exposed to cigarette smoke.³

Although cell-cycle delay and apoptosis induction are quite efficient in defending the foetus against ODD, their consequence is the occurrence of hypoplasia resulting in a delayed development of the whole foetal organism or of selected organs. Accordingly, transplacentally smoke-exposed mouse foetuses undergo a significant decrease of body weight at birth and an underdevelopment of the whole organism with an increased probability of abortion and subsequent parity decrease.³ These findings explain, at a molecular level, the results of epidemiological studies unequivocally demonstrating that smoke-exposure during pregnancy results in retardation of intrauterine growth, decreased newborn weight, and delivery of small for gestational age babies.¹¹ The delayed development may also occur only in selected organs and tissues, mainly including bones and the nervous system. In the first case, facial bones are often affected, transplacental smoke-exposure resulting in an increased risk of orofacial cleft.¹² In the second case, the respiratory centre of the spinal-marrow bulb (*medulla oblongata*) may be affected, this situation resulting in an increased risk of sudden infant death syndrome.¹³

The Foetus-Newborn Transition

Physiological Situation

As previously reported, the level of ODD in the unexposed foetus is particularly low under physiological conditions compared to other life stages. However, a sudden and dramatic change occurs at birth, characterising at a functional and molecular level the foetus-newborn transition. At delivery, the newborn acquires independent respiratory function, which induces relevant physiological and anatomical changes in the lung. As a result the alveoli, physiologically collapsed and atelectatic during intrauterine life, are suddenly expanded and filled with air at delivery. These changes occur in only a few seconds, with no time to efficiently activate the antioxidant defence mechanisms in the newborn, which therefore undergoes a sudden and dramatic oxidative burden. It is well known that the oxygen partial pressure in blood undergoes a sudden increase at the moment of the delivery,¹⁴ resulting in increased ODD formation during the foetus-newborn transition. This is a physiological situation occurring in absence of any exposure to exogenous genotoxic agents. These findings have been demonstrated by monitoring ODD in mouse foetuses 24 h before delivery and immediately after birth. Significant ($p < 0.05$) increases of 8-OH-dG (1.9-fold) and lipophilic bulky DNA-adducts (5.0-fold) were observed in mouse lung during the foetus-newborn transition.² We compared perinatal ODD in mouse lung,² heart, and liver (Izzotti et al unpublished data) in terms of 8-OHdG and lipophilic bulky DNA-adduct formation (Fig. 4). Lung was the only organ undergoing a significant 8-OHdG increase, while bulky DNA-adducts, as related to the basal metabolic rate, were significantly increased not only in the lung but also in the liver (3.96-fold). This finding reflects the involvement of the liver in the activation of anti-oxidant defences, as supported by the transient increase (up to 20-fold) of oxidized glutathione through the foetal-natal transition, followed a few hours after birth by a remarkable (6-fold) increase of reduced glutathione synthesis, as observed in rat.¹⁵

Newborn lung promptly counteracts perinatal ODD by activating the expression of genes involved in antioxidant defence pathways, including glutathione-related genes, catalase, and

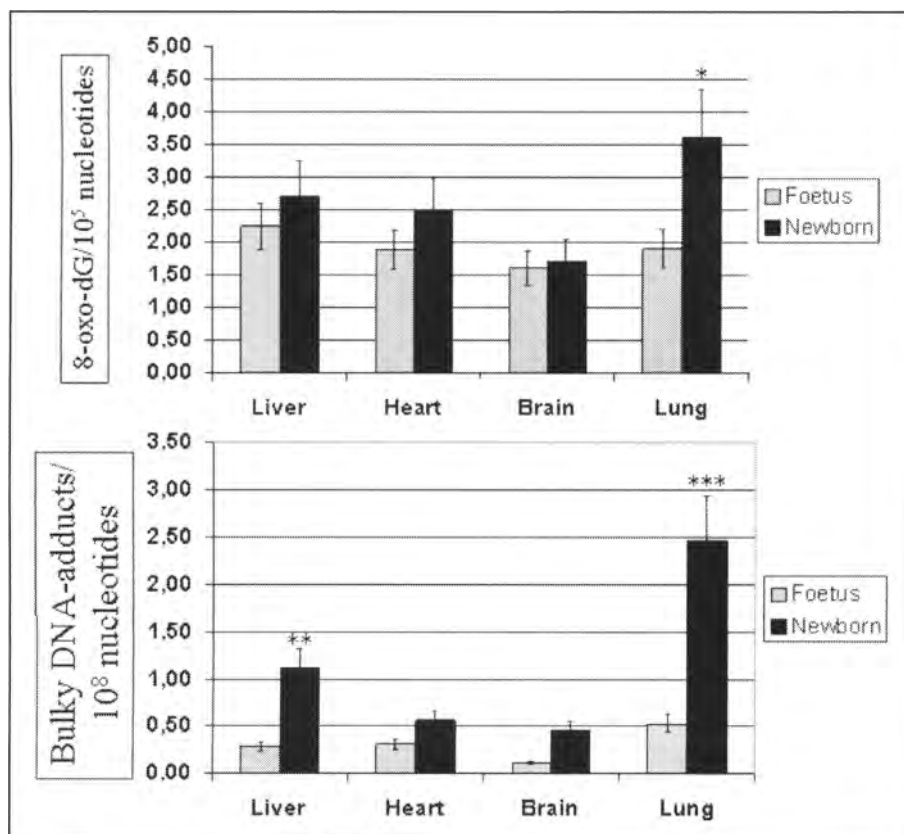


Figure 4. Increase of oxidative DNA damage as observed during the foetus-newborn transition in mouse organs. A significant ($*P < 0.05$) increase of 8-hydroxy-2'-deoxyguanosine (8-oxo-dG) was detected in the lung (upper panel). Significant increases of lipophilic bulky DNA adducts were detected in liver ($**P < 0.01$) and lung ($***P < 0.001$) (lower panel).

oxidative-stress induced proteins.² Therefore, the consequence of oxidative stress occurring in the lung during the foetus-newborn transition is the activation of antioxidant pathways currently used during autonomous respiration. Under these conditions, perinatal oxidative stress may be considered a physiological situation contributing to the development and maturation of the respiratory apparatus. This interpretation is confirmed by the finding that genes encoding for DNA-repair related activities (e.g., endonuclease III, DNA topoisomerase I) are silent during the intrauterine life but become transcriptionally active immediately after birth in mouse lung.²

Pathological Conditions: Antioxidant Deficiency

It may not be excluded that, whenever perinatal oxidative stress in the lung quantitatively exceeds the physiological level, it may have adverse consequences on respiratory function. This condition may be realised whenever an imbalance, compared to the physiological condition, between oxidants and antioxidants occurs in the pregnant mother. A decreased level of antioxidant defences may occur in the newborn as a consequence of maternal nutritional insufficiency during pregnancy. Furthermore, it has been demonstrated that the consumption of pro-oxidant substances, such as iron, during pregnancy, may abnormally increase the intensity of perinatal

oxidative burst in the rat, with concomitant increases in bulky oxidative adducts.¹⁶ It has been suggested that exceedingly high levels of oxidative damage in the lung of newborns may be a pathogenic factor for the occurrence of broncopulmonary dysplasia¹⁷ or childhood lymphocytic leukaemia,¹⁸ although the underlying mechanism remains to be clarified.

Interestingly, the addition of antioxidants to the maternal diet during pregnancy has been shown to decrease the intensity of the perinatal oxidative burst, as demonstrated by administering *N*-acetylcysteine, a glutathione precursor, to mouse dams.² Therefore, the balance between oxidants and antioxidant nutrients during pregnancy appears to be an important factor determining the physiological or pathological meaning of the perinatal oxidative burst.

Intermediate Life Stages

Physiological Situation

Progressive accumulation of ODD occurs during the intermediate life stages, as demonstrated in a variety of experimental animal models¹ and humans.¹⁹ This situation reflects physiological consequences of aerobic metabolism, resulting in ODD accumulation in different organs in a time-dependent manner. Under physiological conditions, this accumulation is moderate and occurs with a linear trend during this period of life (Figs. 1 and 2).

Pathological Conditions: Chemical, Physical and Biological Oxidising Agents

The induction of high levels of ODD can result from exposure to many pathological agents, such as environmental oxidising agents with a chemical, physical and also biological nature. The availability of microarray methods enables exploration of these situations by revealing an extraordinary high degree of molecular detail.

As examples for chemical oxidizing-agents, the consequences of ODD at genomic, postgenomic, and proteomic levels have been investigated in detail in experimental animal models describing the multiple consequences of exposure to established human carcinogens, e.g., hexavalent chromium^{20,21} and cigarette smoke.²²⁻²⁴ These studies demonstrate that ODD induced by cigarette smoke represents a risk factor for the occurrence not only of lung cancer but also of other inflammatory and chronic-degenerative diseases including asthma, emphysema, and stroke.

Physical oxidizing-agents such as UV-containing light induce, in mouse skin, multiple alterations of gene expression, as analysed by cDNA array, amenable to a variety of pathological conditions including cancer, skin ageing, angioproliferation, and immunosuppression.²²

Furthermore, it has been demonstrated, by analysing ³²P-postlabelled lipophilic bulky DNA-adducts, that light exposure also induces ODD in distant organs, including bone marrow and lung,²⁵ and triggers the expression of genes encoding anti-oxidant activities, i.e., glutathione transferase P and catalase, in the lung.²² In the case of involvement of haematopoietic organs, light-induced ODD is likely to represent a possible risk factor for leukemias and lymphomas, as suggested by animal studies²⁶ and human epidemiological studies.²⁷

Infective agents may also represent an important source of ODD. It has been demonstrated, in an experimental animal model of viral hepatitis-B infection, that this situation results in a significant increase of lipophilic bulky DNA adducts paralleled by a significant failure of the antioxidant defences, as indicated by the decreased availability of reduced glutathione.²⁸ In these circumstances, formation of ODD represents a pathogenic mechanism favouring liver cancer development.

ODD as related to infective agents, is also demonstrated to occur in the human gastric mucosa of *Helicobacter pylori* infected patients.²⁹ However, ODD is significantly influenced by the genetic features of both *Helicobacter pylori* and the host organism. In fact, the highest levels of ODD were detected only in cases of infection with *cagA*+ *Helicobacter pylori* strains (Izzotti et al unpublished data).

Late Life Stages

Physiological Situation

During the late life stages ODD dramatically increases (Figs. 1 and 2). This situation is not only the result of the progressive ODD accumulation during many years, but also underlies the steady overwhelming of antioxidant defence mechanisms and their consequent functional failure, as demonstrated in animal experimental models¹ and humans.¹⁹ However this phenomenon occurs in a different manner in various organs (Fig. 2). The detection of ODD in mouse in terms of 8-OHdG, bulky DNA adducts and DNA-protein crosslinks, significantly increases with age in brain and, particularly in the heart, while a less consistent increase is observed in liver.¹ These findings suggest that ODD gradually and irreversibly accumulates in heart and brain, while antioxidant defence mechanisms render the liver less susceptible to age-related ODD. The progressive ODD accumulation underlies a physiological decrease of heart and brain performances resulting in age-related degenerative phenomena. Cell turnover is a crucial factor conditioning the pathological consequences of ODD which, when occurring in proliferating epithelia contribute to the development of cancer, and when occurring in perennial-cell populations to the development of degenerative diseases.³³

Pathological Conditions: Atherosclerosis and Glaucoma

The accumulation of ODD as occurring in specific organs may contribute to the appearance of age-related chronic degenerative diseases. This is typified by atherosclerosis, as demonstrated by the detection in the aorta of atherosclerotic patients of remarkable oxidative-related alterations including 8-OHdG, lipophilic bulky DNA-adducts, malondialdehyde, etheno DNA-adducts, common mitochondrial 4977 deletion, accompanied in plasma by homocysteine increase and glutathione decrease (Izzotti et al unpublished data).³⁰⁻³² Levels of 8-OHdG detected in atherosclerotic lesions were higher than those routinely reported in other healthy human tissues and diseased tissues including blood leukocytes from Fanconi anemia, rheumatoid arthritis, systemic lupus erythematosus, Bechet's disease, liver tissue from hepatitis-B infected patients, brain tissue from Alzheimer's patients,³³ and gastric mucosa from *Helicobacter pylori* infected patients. In atherosclerotic patients, 8-OHdG levels were found to be significantly higher (2.75-fold; $p < 0.001$) in the inner than in the medium aorta layer (Izzotti et al unpublished data). A variety of factors are thought to contribute to the formation of such a high level of ODD, including peroxidation of lipid deposits occurring in the aortic layers during atherogenesis and inflammatory processes. Cigarette smoke also plays a major role in inducing ODD in human atherosclerosis, as inferred by (a) the significant relationship between the number of cigarettes smoked per day and the level of malondialdehyde in the aorta; (b) the significant increase of etheno-DNA adducts in the aorta of smokers as compared to nonsmokers; (c) the significant decrease of plasma reduced-glutathione in smokers as compared to nonsmokers.

One other factor significantly contributing to the presence of ODD in atherosclerotic patients is dietary habits. Subjects having a low consumption of fresh fruit and vegetables undergo a significant increase in plasma homocysteine, a major risk factor for atherosclerosis. Homocysteine, an established oxidising agent,³⁴ displays its atherogenic effect through oxidative mechanisms, as suggested by the significant correlation detected between plasma homocysteine and bulky-adducts detected in the aorta by ³²P-postlabelling. Interestingly, the same adduct was also significantly ($p < 0.001$) related to 8-OHdG and triglyceride levels.³⁰ Antioxidant chemopreventive agents were demonstrated to decrease 8-OHdG and lipophilic bulky DNA adduct formation in the thoracic aorta of cigarette-smoke exposed rats.³⁵

Recently, it has been demonstrated that ODD has an important pathogenic role in the main cause of irreversible blindness worldwide, i.e., primary open angle glaucoma. A 3.3-fold increase in 8OHdG increase was observed in patients, compared to unaffected controls³⁶ in the trabecular meshwork, the specialised tissue of the anterior chamber of the eye devoted to the regulation of aqueous humor outflow. Furthermore, high ODD levels were significantly

Table 1. Possible physiological and pathological consequences of oxidative DNA damage (ODD) at different life stages, as discussed in the text

Life Stage	ODD Cause	Pathogenic Mechanism	Possible Consequence	Physiological Consequence
Foetus	Cigarette smoke exposure	Hypoplasia Inflammation	Intrauterine growth retardation Orofacial clefts Sudden infant death syndrome	
Newborn	Perinatal oxidative burst	Macromolecule oxidation in Lung		Activation of antioxidant mechanisms needed for autonomous respiration
	Exceedingly high perinatal oxygen supplementation	Systemic DNA damage	Bronchopulmonary dysplasia Childhood lymphocytic leukemia	
Adult	Endogenous production of reactive oxygen species	Accumulation of DNA damage in perennial cells		Ageing
	Exposure to chemical oxidizing agents through inhalation	Damage to lung DNA	Lung cancer	
	Exposure to physical oxidizing agents (light)	DNA damage	Skin ageing, cancer, angioproliferation, immunosuppression	
	<i>Helicobacter pylori</i> and Hepatitis B virus infections	DNA damage	Gastric cancer Liver cancer	
Senescent organism	Endogenous production of reactive oxygen species	DNA-damage accumulation in perennial cells		Heart and brain age-related degeneration
	Oxidative damage resulting from endogenous and exogenous sources	DNA damage in vascular cells	Atherosclerosis	
	Oxidative damage resulting from endogenous sources	Accumulation of DNA damage in the specialised ocular epithelia regulating aqueous humour outflow	Open angle degenerative glaucoma	

($p < 0.01$) related to clinical variables including increased intraocular pressure and worsening of visual field damage.³⁷ The imbalance between pro- and anti-oxidant activities in primary open angle glaucoma is also indicated by the finding that the homozygous deletion of the glutathione S-transferase M1 gene, which encodes a pivotal antioxidant enzyme, is significantly more frequent in affected patients than in controls.^{36,38,39} As a consequence, various studies are currently exploring the possibility of using antioxidant drugs in therapy and prevention of degenerative glaucoma. Interestingly, timolol, a beta-blocker drug commonly used in glaucoma therapy, was demonstrated to exert antioxidant effects in vitro on human endothelial cells undergoing oxidative stress (Izzotti et al unpublished data).

Conclusions

Reported studies support the view that ODD is a common phenomena occurring under a variety of both physiological and pathological conditions during the whole lifespan of aerobic organisms. ODD may alternatively possess a physiological meaning or have pathological consequences depending on multiple factors including (a) the life stage in which it occurs; (b) the affected organ; (c) the factors causing ODD formation; (d) the quantitative ODD level. As summarized in Table 1, the interplay among all these factors determines whether or not ODD may have pathological meaning and which diseases may occur as its consequence.

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Analysis of 8-Hydroxy-2'-Deoxyguanosine as a Marker of Oxidatively Damaged DNA in Relation to Carcinogenesis and Aging

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Abstract

Reactive oxygen species (ROS) are well known hazards for living organisms and are believed to be associated with the induction of cancer. ROS induce many forms of oxidative damage to proteins, nucleic acids, and lipids. Therefore, to diagnose or prevent cancer, analyses of oxidative products in patient samples, including tissue, blood, and urine, are very informative. Amongst the products of DNA oxidation, 8-hydroxy-2'-deoxyguanosine (8-OH-dG) is an important form of damage that leads to point mutations in genomic DNA. Since 8-OH-dG is the most abundant form of oxidative DNA damage and is easy to detect in laboratories, using a high performance liquid chromatography (HPLC) system equipped with an electrochemical detector (ECD), many researchers studying cellular oxidative stress have primarily analyzed 8-OH-dG. In this chapter, we will describe our findings regarding 8-OH-dG generation and its repair, as well as our recent urinary 8-OH-dG data.

Introduction

The formation of 8-hydroxy-2'-deoxyguanosine (8-OH-dG) in DNA by ROS in vitro was first reported by Kasai and Nishimura in 1984.¹ Many biochemical studies of this lesion have subsequently been performed. One of the notable characteristics of 8-OH-dG is its mutagenicity. It is well known that 8-OH-dG in nuclear DNA induces GC to TA point mutations, which are believed to be associated with carcinogenesis.² This characteristic of 8-OH-dG supports the significance of 8-OH-dG measurements in DNA for studies of carcinogenesis. Since the first report in 1984, several efforts have been made to promote 8-OH-dG analyses.

- i. The first efforts focused on the development of an improved detection method for 8-OH-dG with reduced background levels, since baseline levels of 8-OH-dG in cellular DNA have been notably different between laboratories. These differences may arise from artefactual formation of 8-OH-dG during DNA isolation, digestion or derivatization prior to analysis. To establish the significance of 8-OH-dG analysis as a biomarker, it is important to standardize the analytical method. Many attempts to decrease the background levels were made

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with antioxidants and metal chelators, and the detection level of 8-OH-dG in the steady state was reduced to one 8-OH-dG residue per 10^6 dG in cultured mammalian cells, using the iron-chelator desferal³ and a commercial DNA extraction kit containing NaI.⁴ As far as we know, the latter DNA isolation method is presently the most reliable one. There is no doubt that a well developed analysis system will be useful to reveal the exact relationship between oxidative DNA damage, carcinogenesis and aging.

- ii. The second question addressed what kinds of carcinogenesis-related factors contribute to the generation of 8-OH-dG, and whether correlations exist between these kinds of carcinogens and the level of 8-OH-dG. The relationship between well-known carcinogens or mutagens and 8-OH-dG generation has been investigated to clarify the carcinogenic mechanism. For example, we reported increased levels of 8-OH-dG in rat and hamster lung DNA after intra-tracheal instillation of crocidolite asbestos.⁵ These results agreed well with our prediction, suggesting that one of the mechanisms of asbestos-induced lung cancer or mesothelioma is 8-OH-dG generation in DNA.
- iii. The third issue addressed the identification of 8-OH-dG repair systems. The integrity of genomic DNA is undoubtedly important to maintain morphological and functional normality of living organisms. One of the factors that impairs the integrity of DNA are ROS. From this point of view, elucidation of the details of 8-OH-dG repair systems is extremely important and useful for understanding the mechanisms by which ROS lead to genomic instability.

Although preliminary 8-OH-dG analyses were performed mainly on cellular DNA, a recently developed technique for the measurement of urinary 8-OH-dG content is now being used to evaluate oxidative stress in living organisms. We have established an accurate method of measuring the urinary 8-OH-dG level by HPLC, using anion-exchange- and reverse-phase columns.⁶ Such measurements may open new fields in the studies of risk assessment, molecular epidemiology, and health promotion. In this chapter, we discuss the significance of measurements of 8-OH-dG levels and its repair ability in relation to carcinogenesis and aging.

Methods for Measuring 8-OH-dG in DNA

In early studies, the background level of 8-OH-dG in cellular DNA was relatively high, due to artificial oxidation of the DNA during the experimental procedure, namely several 8-OH-dG residues/ 10^5 dG, in comparison to the current level (one 8-OH-dG residue/ 10^6 dG). As the measurement of the exact value of 8-OH-dG generation was of special interest, many researchers made efforts to overcome the problem of relatively high background levels of 8-OH-dG. After evaluation of several methods, the method using a commercial DNA extraction kit containing NaI was recognized by Nakae et al⁴ as the most reliable in terms of reducing the background level of 8-OH-dG. Subsequently, it was found that the addition of the iron-chelator desferal³ to the homogenization solution was also useful in preventing artifactual 8-OH-dG formation. Therefore, we now recommend using a combination of these two methods for measuring 8-OH-dG levels in DNA. Briefly, the tissue or cellular sample is homogenized in the presence of desferal (1 mM) by a Potter-type homogenizer. The nuclear DNA is extracted by using the DNA Extractor WB kit (Wako Biochemicals, Osaka, Japan), which contains NaI (iodide ion functions as a hydroxyl radical scavenger in this extraction). After digestion with nuclease P1 and alkaline phosphatase, the sample is analyzed by high performance liquid chromatography (HPLC) equipped with an electro-chemical detector (ECD) (ESA Coulochem II, Chelmsford, MA USA). It is also important to keep the DNA digest in the -80°C freezer until immediately before the HPLC analysis, to prevent artefactual formation of 8-OH-dG (Kawai and Kasai, unpublished data). As with calibration standards, 20 μl aliquots of deoxyguanosine (0.5 mg/ml) and 8-OH-dG (5 ng/ml) solutions are injected. The 8-OH-dG value is calculated as the number per 10^6 2'-deoxyguanosine residues.

Formation of 8-OH-dG in DNA of Animal Organ or Cultured Cell DNA during Aging and by Treatment with Oxidative Stress-Inducing Carcinogens

We previously analyzed the steady state 8-OH-dG levels in rat organ DNA, in association with the aging process. Although we had expected that the generation of 8-OH-dG would increase with the aging process, the 8-OH-dG levels in the rat organs investigated in our study (liver, spleen, intestine, kidney, lung, and brain) did not show any significant change during the aging process from 5 months to 30 months which corresponds to about 30-70 years in human age.⁷

In contrast, experimental inductions of oxidative stress in animal models were also performed to investigate the relationship between 8-OH-dG generation and cancer. We used chemical compounds that are known to induce cancer in their target organs, such as ferric nitrilotriacetate, crocidolite asbestos, cadmium chloride, diesel exhaust particles, and arsenic compounds.^{5,8-10} It was found that all of these oxidative stress-inducing carcinogens increased 8-OH-dG levels in the target organ DNA, suggesting a significant role for ROS in carcinogenesis. In addition, to study the role of ROS or ROS-induced disorders in carcinogenesis, the generation of 8-OH-dG in the DNA of cultured cells treated with chemical agents was also measured. For example, we analyzed the effects of an arsenic compound on the human lung carcinoma cell line, A549, and found that it increased the 8-OH-dG level in the cellular DNA.¹²

We analyzed the steady state level of 8-OH-dG in the cultured human fibroblast cell line, TIG-3, in relation to aging.¹¹ In contrast to the animal experiments, the 8-OH-dG level increased with the cellular aging process.

Clinical Analysis of 8-OH-dG in Human DNA

To evaluate the utility of measuring the 8-OH-dG level in human DNA as a clinical analysis tool, we analyzed leukocyte or tissue DNA samples obtained from surgery patients.¹³⁻¹⁶ For example, we observed increased levels of 8-OH-dG in the leukocyte DNA of smokers as compared to nonsmokers.¹³ In this study, we investigated the levels of 8-OH-dG in leukocyte DNA obtained from 10 current smokers, 10 ex-smokers, and 10 complete nonsmokers. We found that the level of 8-OH-dG was significantly ($p = 0.013$) increased by cigarette smoking, suggesting that measuring the 8-OH-dG level in leukocyte DNA is useful to estimate the effect of cigarette smoking on human DNA or to carry out a risk assessment of smoking, without the analysis of lung tissue DNA. A linear relationship was obtained between 8-OH-dG levels and the number of cigarettes smoked per day ($r = 0.62$; $p = 0.002$).

In the experiments using the samples obtained from surgery, the 8-OH-dG levels in the peripheral parts of human lung tissues were compared between lung cancer patients ($n = 70$) and noncancer patient controls ($n = 15$).¹⁵ A significantly increased ($p < 0.01$) level of 8-OH-dG was observed in the lung cancer group. We also analyzed the 8-OH-dG levels in colorectal biopsy samples from the normal tissue of patients with either colorectal cancer ($n = 15$) or benign colorectal polyps ($n = 40$).¹⁶ However, in that study, we did not observe a significant change in the 8-OH-dG level in the DNA of the patients with cancer as compared to those with polyps, although an age-associated increase of 8-OH-dG was observed.

Analysis of the 8-OH-dG Repair System

Immediately after the discovery of 8-OH-dG, repair enzymes for 8-OH-dG were predicted to exist, and many research groups attempted to detect and clone them. Before the gene encoding the 8-OH-Gua-specific repair enzyme was cloned, we employed an endonuclease nicking assay using a ³²P-labeled 22 mer double-stranded synthetic oligonucleotide containing

8-OH-Gua, to analyze the base excision repair activity for 8-OH-Gua. By using this method, we investigated the relationship between 8-OH-dG formation, repair and cellular aging.¹⁷ As a result, we found decreased levels of 8-OH-Gua repair activity during the latter part of cellular aging, suggesting an inverse relationship between 8-OH-Gua formation and 8-OH-Gua excision repair activity.

In 1997, the genes encoding the human and mouse 8-OH-Gua glycosylase-type repair enzymes, *hOGG1* and *mOGG1* respectively, were cloned by several research groups.¹⁸⁻²³ This repair protein has two enzymatic activities, glycosylase and AP lyase.²³ We analyzed the lung *OGG1* mRNA expression of rats intra-tracheally exposed to diesel exhaust particles (DEP).¹⁰ We detected the inhibition of *OGG1* mRNA expression in the lungs of the DEP-exposed rats in comparison to the controls, suggesting that the inhibitory effect of DEP on the DNA repair system might be related to the carcinogenic mechanisms of DEP exposure. Thus, analyses of *OGG1* mRNA expression might be useful to study carcinogenic mechanisms.

More recently, we detected a 32-kDa mOGG1 fragment in the livers of mice fed a diet containing 0.06 % 3'-methyl-4-dimethylaminoazobenzene²⁴ (Fig. 1A) and in mouse nonparenchymal hepatocytes (NCTC) treated with etoposide or mitomycin C (MMC)²⁵ (Fig. 1B). It is noteworthy that, in both cases, 8-OH-Gua accumulation was observed in parallel with mOGG1 cleavage. In addition, we confirmed that mOGG1 fragmentation was related to the apoptotic process, because the 32-kDa mOGG1 fragment disappeared when a caspase inhibitor was added to the medium²⁵ (Fig. 1C). In summary, mOGG1 was cleaved during the apoptotic process which, in turn, caused a reduction in the 8-OH-Gua repair activity, leading to 8-OH-Gua accumulation. However, the precise mechanism of OGG1 fragmentation remains unknown.

Analysis of Urinary 8-OH-dG

Urinary 8-OH-dG is a good indicator of oxidative stress in vivo. Patients with cancer and other oxygen radical-related diseases have high urinary 8-OH-dG levels.^{26,27} The 8-OH-dG levels in urine and lymphocyte DNA are also well correlated, and the 8-OH-dG/creatinine values of 24 hr urine samples and overnight urine (early morning urine) show a good correlation.²⁸

In our urinary 8-OH-dG analysis method,⁶ an anion exchange column (polystyrene-type resin with a quaternary ammonium group, sulfate form) is used for a urine prepurification step (HPLC-1), which removes most of the other components in the sample. In HPLC-1, the 8-OH-dG fraction is accurately collected based on the elution position of the ribonucleoside 8-hydroxyguanosine, added as a marker (Fig. 2A). Furthermore, by monitoring two ECD channels with different applied voltages, for example, 170 and 300 mV, the 8-OH-dG peaks appear with a specific ratio of peak heights, as shown in Figure 2B, which is useful to confirm the peak purity.

Creatinine (Cre), a measure of urine concentration, is frequently used to normalize urinary 8-OH-dG values. We attempted to analyze both 8-OH-dG and Cre simultaneously in the HPLC-1 step with our method.²⁹ When we chose 245 nm for monitoring in HPLC-1, and used a thinner UV cell, we successfully detected the Cre peak, as shown in Figure 2A. It was also possible to measure 7-methylguanine, a product of DNA methylation, by monitoring 305 or 245 nm in addition to Cre.³⁰

To clarify the relationship between lifestyles and oxidative DNA damage, the urine samples of 372 employees of a company were analyzed.³¹ Alcohol drinking, cigarette smoking, average working hours and serum cortisol, a stress hormone, showed positive correlations with the 8-OH-dG level, while BMI, consumption of soybean products, rice and light-colored vegetables showed negative correlations (Table 1). One of the mechanisms for the increase of 8-OH-dG by stress may be the generation of ROS by catecholamine metabolism.³²

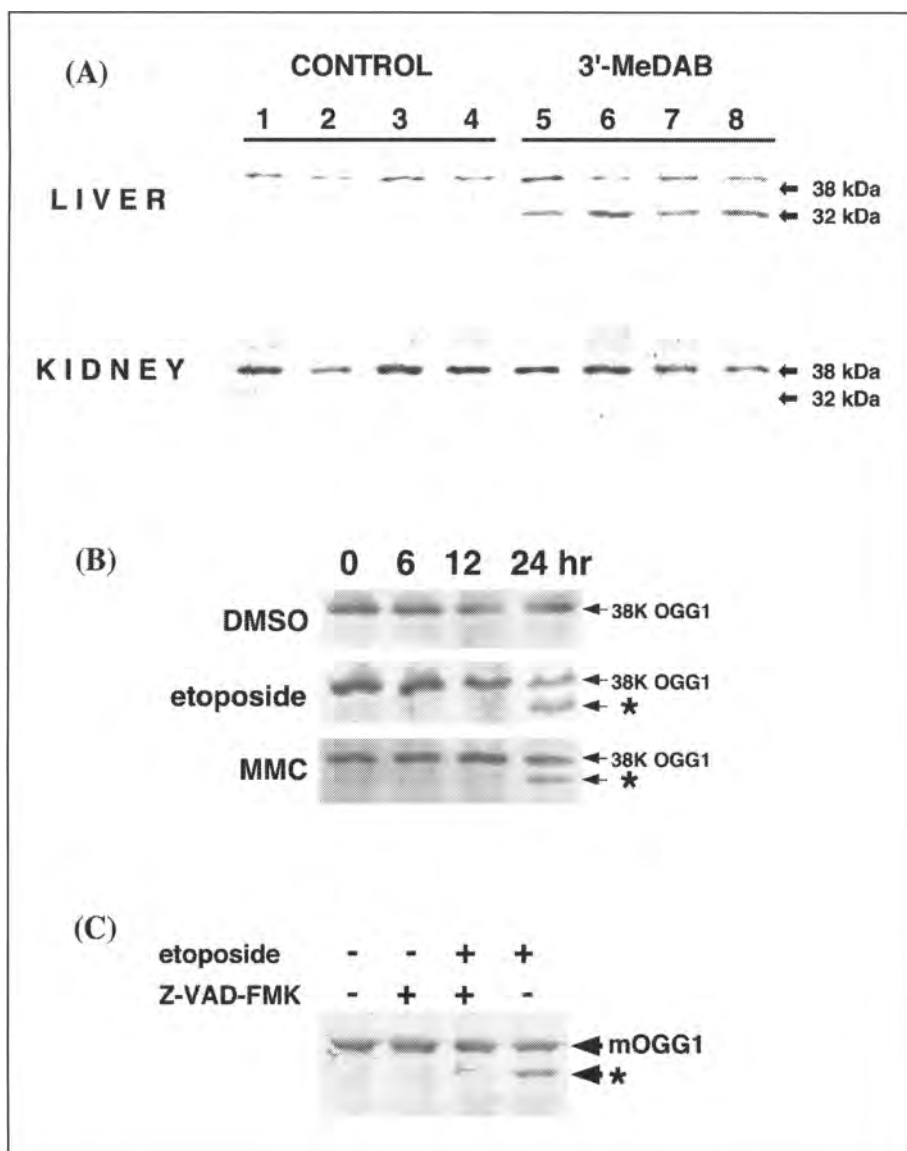


Figure 1. Detection of a mOGG1 fragment. A) Immunoblotting of mOGG1 in the livers and kidneys of the 3'-methyl-4-dimethylaminoazobenzene (MeDAB)-treated mice. Two clear bands at the positions of 32- and 38-kDa are seen only in the 3'-MeDAB-treated mouse livers. B) mOGG1 expression in the nonparenchymal hepatocytes (NCTC) treated with DMSO (top), 100 μ M etoposide (middle), and 30 μ M mitomycin C (MMC) (bottom) at 0, 6, 12, and 24 h of cultivation. Cleaved mOGG1(*) is visible. C) Effect of a caspase inhibitor on mOGG1 expression. The immunoblotting analysis failed to detect the mOGG1 fragment (*) in the etoposide-treated NCTC grown in the presence of Z-VAD-FMK.

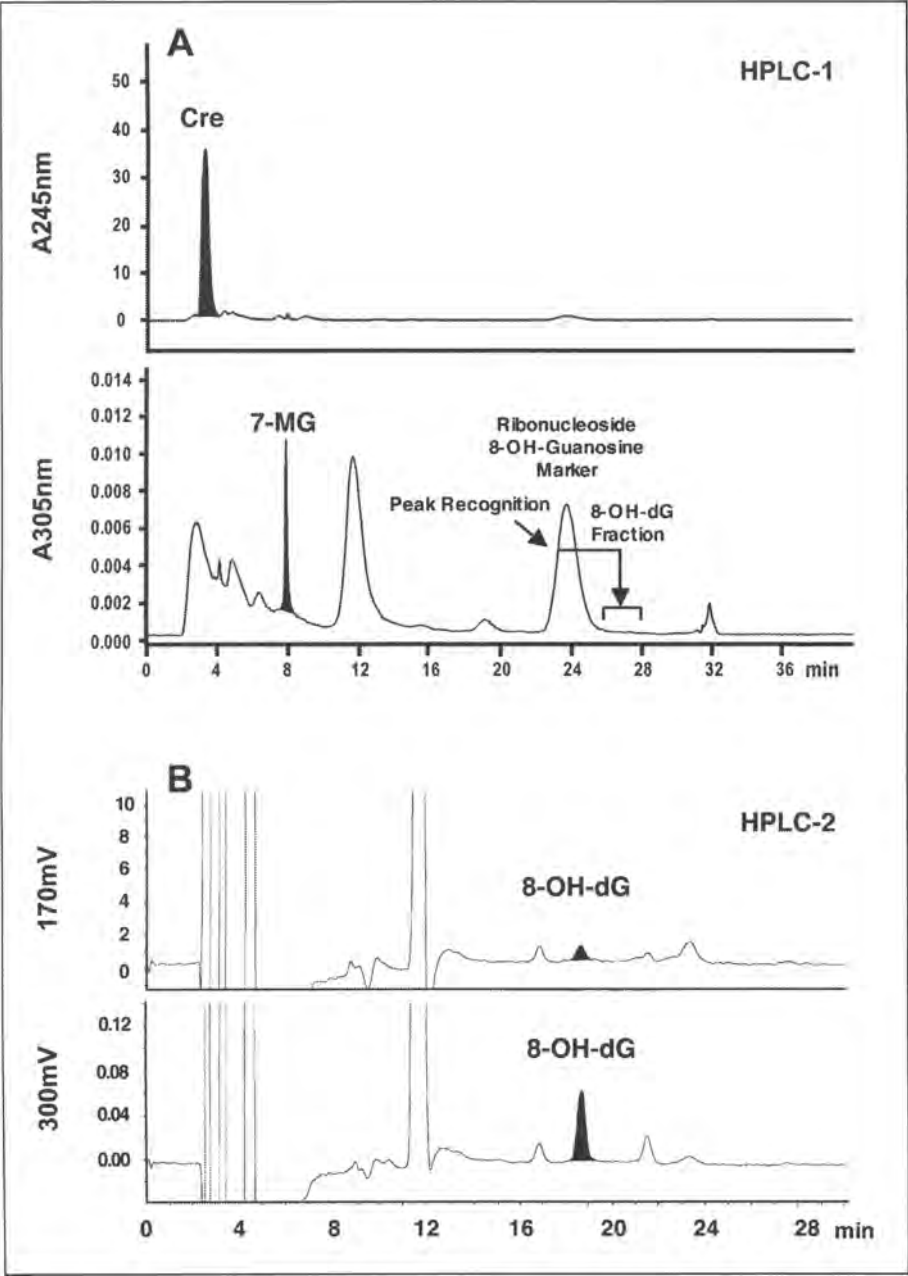


Figure 2. Analyses of creatinine (Cre), 7-methylguanine (7-MG) and 8-OH-dG by two step-HPLC. A) Detection of Cre and 7-MG by anion exchange chromatography (HPLC-1). B) Detection of 8-OH-dG by reverse-phase HPLC (HPLC-2).

Table 1. Spearman's correlations between lifestyle variables and urinary 8-OH-dG levels

	Male (n = 286)	Female (n = 86)	Total (n = 372)
Age	-0.10	0.29**	-0.06
BMI	-0.23**	-0.26*	-0.20**
Work hours	0.19**	-0.08	0.21**
Smoking	0.18**	0.24*	0.28**
Alcohol drinking	0.14*	0.09	0.17**
Soybean products	-0.09	-0.17	-0.13*
Intake of rice	-0.14*	-0.16	-0.13*
Light-colored vegetables	-0.07	-0.16	-0.13*
Serum cortisol	0.10	0.18	0.19**

* $p < 0.05$, ** $p < 0.01$

Urinary 8-OH-dG Levels in Cancer Patients and Cancer High-Risk Groups

The urinary 8-OH-dG levels of cancer high-risk patients³³ (dermatomyositis, polymyositis, systemic sclerosis), cancer suspected disease (CREST syndrome, Sjögren's syndrome), cancer nonassociated diseases (rheumatoid arthritis, systemic lupus erythematosus), and extremely old people (volunteers in a nursing home, mean age was 81 years old) were determined by the method described above.³⁴ In addition, the urinary 8-OH-dG levels of cancer patients before/after surgery were also measured. As a control for these groups, the urinary 8-OH-dG levels of healthy volunteers, with a mean age of 36 years, were determined.

The 8-OH-dG levels of the cancer high-risk group (9.29 ± 5.19 ng/mg creatinine, $n = 19$), the cancer suspected group (7.75 ± 2.99 , $n = 3$), and the cancer patients (before operation, 7.15 ± 4.01 , $n = 103$; after operation, 8.32 ± 5.13 , $n = 98$) were significantly higher as compared to that of the healthy control group (4.12 ± 1.73 , $n = 323$). Furthermore, the 8-OH-dG levels of very old people (6.01 ± 2.93 , $n = 35$) were also significantly higher than those of the control group, while the patients with noncancer-associated diseases (4.81 ± 1.78 , $n = 11$) showed 8-OH-dG levels similar to those of healthy people.

In conclusion, the 8-OH-dG levels in urine were higher in patients with cancer-associated diseases and in extremely old people, as compared to the levels in healthy people, suggesting that oxidative DNA damage might be involved in cancer development and the aging process. Therefore, analyses of the urinary 8-OH-dG levels are useful and informative for cancer research.

Discussion

In this chapter, we have discussed our findings based on the studies of 8-OH-dG performed so far in our laboratory. As we described above, to evaluate the effect of ROS on living organisms, measurements of the 8-OH-dG levels and its repair capacity are a useful and powerful strategy, particularly to understand carcinogenic mechanisms, to assess the carcinogenic risk of environmental factors, and to open a new approach to diagnosis of cancer. One important point to consider when studying 8-OH-dG is that the 8-OH-dG level is the consequence of the balance between 8-OH-dG generation and repair. Therefore, measurements of 8-OH-dG without analyses of its repair ability are not sufficient to understand the mechanisms underlying increased/decreased levels of 8-OH-dG. For example, although cadmium chloride is not thought to induce ROS directly, because Cd^{2+} is not capable of accepting or donating electrons

under physiological conditions,³⁵ 8-OH-dG was increased in the testes DNA of rats treated with CdCl₂.⁹ The explanation for this result is that the increased level of 8-OH-dG was due to the inhibition of repair activity. A similar conclusion, that the 8-OH-dG increase in DNA by Cd²⁺ is due to impaired 8-OH-dGTPase activity, was previously reported by Kasprzak and his collaborators.³⁶ In this context, analyses of both 8-OH-dG levels and its repair (OGG1 and 8-OH-dGTPase) activity are required for accurate investigations.

To understand the mechanisms of carcinogenesis and other ROS-related diseases, further studies of 8-OH-dG production and repair are required. In addition to the investigations of disease mechanisms, measurements of 8-OH-dG may be useful for understanding the pathogenesis of ROS-related diseases and hence suggest intervention strategies and treatments. For example, patients with chronic hepatitis (urine),³⁷ diabetes (urine, leukocyte DNA),^{38,39} heart disease (leukocyte DNA),⁴⁰ Alzheimer's disease (urine),⁴¹ Parkinson's disease (urine),⁴² and atopic dermatitis (urine),⁴³ as well as premature babies (urine),⁴⁴ showed higher levels of 8-OH-dG. Therefore, 8-OH-dG is a useful marker for monitoring the cellular oxidative stress involved in the induction of cancer and ROS-related diseases.

In conclusion, since we use oxygen molecules as a source of energy, we will never be able to avoid oxidative damage to proteins, lipids, and nucleic acids. Since there is a growing body of evidence that these forms of oxidative damage contribute to many kinds of diseases, the 8-OH-dG level in DNA or urine can serve as a useful biomarker of ROS production in these diseases. In this context, 8-OH-dG studies are undoubtedly useful for cancer and ROS-related disease research.

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CHAPTER 15

Oxidatively Damaged DNA and Inflammation

Peter C. Dedon* and Marita C. Barth

Abstract

Chronic inflammation has long been associated with diseases such as cancer and cardiovascular disease, with infectious processes playing a causal role in many types of cancer. One potential link between chronic inflammation and human disease involves the release of highly reactive oxygen and nitrogen species by macrophages and neutrophils in inflamed tissues. In addition to their intended targets, these species also attack surrounding host tissue cells, with damage to virtually all cellular components, including DNA, RNA, proteins, carbohydrates and lipids. This chapter addresses the role of inflammatory processes in DNA damage in cells and tissues, whether by direct attack on DNA or by indirect mechanisms involving generation of electrophiles that subsequently react with DNA to form adducts.

Introduction

Epidemiological studies have demonstrated a strong association between chronic inflammation and increased cancer risk,¹⁻⁴ such as the relationship between inflammatory bowel disease and colon cancer,^{5,6} *Helicobacter pylori* infection and gastric cancer,^{7,8} and *Schistosoma haematobium* infection and bladder cancer.^{9,10} One potential link between inflammation and disease lies in the infiltration by macrophages and neutrophils at sites of inflammation and release of chemically reactive species intended to bring about the elimination of infectious agents.^{11,12} Amongst these chemical mediators of inflammation are the reactive oxygen, nitrogen and halogen species shown in Figure 1. These same inflammatory mediators can also damage surrounding host tissue, leading to pathological reactions with cellular components that include polyunsaturated fatty acids, proteins, carbohydrates and nucleic acids. Whilst there are a variety of nongenotoxic mechanisms participating in the pathophysiology of chronic inflammation, including resistance to apoptosis,^{13,14} cytotoxicity and compensatory hyperproliferation,¹⁵ adaptive changes,¹⁶ and enhanced angiogenesis,¹⁷ inflammation-induced DNA damage, caused by direct reaction with chemical mediators of inflammation or indirectly by DNA reactions with electrophiles generated from other molecules, still holds a central position in the scheme of somatic mutagenesis that results in malignant transformation.^{18,19} However, recent studies suggest that not all of the chemistries associated with inflammation play a role in generating DNA damage. This review will focus on the chemistry of inflammation-induced DNA damage based upon predictions made from in vitro studies and the realities of the chemistry occurring in inflammatory cells and inflamed tissues.

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Chemical Mediators of Inflammation

The chemical mediators of inflammation shown in Figure 1 cause a wide range of reactions, including nitrosation, nitration, oxidation and halogenation. While neutrophils play an important role in inflammation, macrophage-derived nitric oxide (NO) represents one of the fundamental mediators of the small molecule chemistry of inflammation, with strong evidence for a role in the cytotoxic and mutagenic mechanisms in the carcinogenesis associated with chronic inflammation.^{1,2,20}

Nitric oxide is synthesized by three different NO synthases and, at low levels (nanomolar), is essential as an endogenous regulator of the cardiovascular, nervous, and immune systems.²¹⁻²⁷ However, long-term overproduction of NO , and its derivatives, by stimulated macrophages, leads to concentrations approaching $1 \mu\text{M}$ ²⁸⁻³⁰ and to generation of reactive species capable of oxidation, nitration and nitrosation. In chronically inflamed tissue, high local concentrations of NO are available for reaction with oxygen or superoxide to generate a multitude of reactive species, as shown in Figure 1. While the biological effects of NO ultimately depend on the complexity of the local cellular milieu and the diffusion distances between generator and target cells,³¹ the reactions of NO can be classified along three general lines: (1) diffusion and intracellular consumption by glutathione and other chemical and enzymatic species; (2) auto-oxidation to form nitrous anhydride (N_2O_3 ; Fig. 1); and (3) reaction with superoxide to form peroxynitrite, ONOO^- , that can react further with CO_2 to form nitrosoperoxycarbonate (ONOOOCO_2^- ; Fig. 1).³²

While the competing chemistries of oxidation and nitrosation occur at sites of inflammation, the short half-lives of the reactive chemical mediators of inflammation lead to difficulties in studying their behavior *in vivo*. To this end, there has been a significant effort to develop biomarkers as surrogates for the reactive oxygen and nitrogen species to define the roles of these species in inflamed tissues, with a focus on DNA lesions, given their potential participation in the carcinogenic processes associated with chronic inflammation.

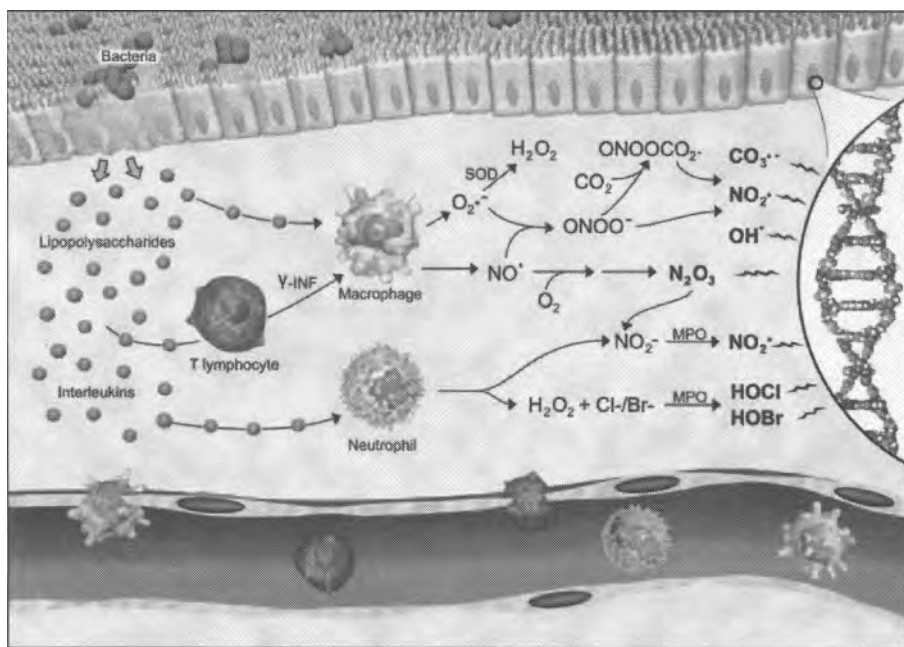


Figure. 1. Inflammation biology and chemistry concepts. Illustration by Jeff Dixon (www.jeffdixon.ca).

Nitrosative Deamination of DNA

The autooxidation of $\cdot\text{NO}$ produces N_2O_3 , which is presumably the primary nitrosating species arising at sites of inflammation.³⁰ The nitrosation of primary amines in DNA and RNA bases, as well as lysine side chains in proteins, leads to their replacement with hydroxyl groups or to cross-linking reactions. As shown in Figure 2, exposure of DNA to N_2O_3 leads to the conversion of cytosine to uracil (2'-deoxyuridine; dU), guanine to either xanthine

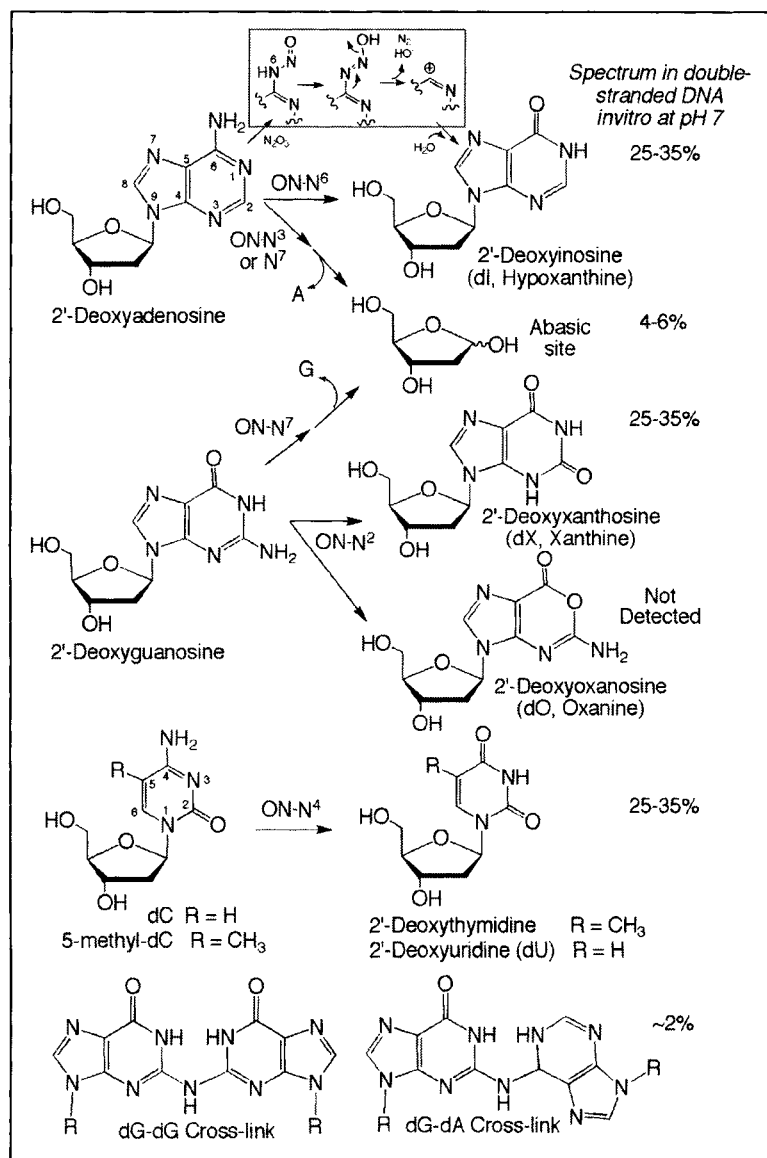


Figure 2. Chemistry of nitrosative deamination of DNA. As illustrated in the boxed inset, nitrosation of the different nitrogen positions in the purine and pyrimidine nucleobases (see ring numbering; NO-N^x notations) leads to deamination of the bases and replacement of the nitrogen with water.

(2'-deoxyxanthosine; dX) or oxanine (2'-deoxyoxanosine; dO), and adenine to hypoxanthine (2'-deoxyinosine; dI), in addition to the formation of abasic sites by depurination and the formation of dG-dG and dG-dA cross-links.^{20,33}

The chemistry of nitrosative deamination of nucleobases in DNA has proven to be both complicated and experimentally challenging to define. The simplest mechanism, and the one likely to account for formation of dU from dC and dI from dA, involves nitrosation of exocyclic amines in the nucleobases with subsequent nucleophilic substitution of N₂ by water. The observed formation of abasic sites likely involves similar N-nitrosation of the N7 positions of dG and dA.³³⁻³⁵ However, the formation of both dX and dO from nitrosative deamination of dG obviates the generally accepted model of a simple guaninediazonium ion intermediate common to xanthine and G-G cross-links. Suzuki et al originally described the formation of dO in reactions of DNA with nitrite under acidic (pH<4) conditions^{36,37} and Shuker and coworkers observed the formation of oxanine base in reactions of DNA with millimolar concentrations of weakly buffered 1-nitrosoindole-3-acetonitrile.^{34,35} These observations stand in contrast to our inability to detect dO using sensitive LC-MS and LC-MS/MS methods to quantify it in DNA exposed to [•]NO and O₂ in vitro,³³ in DNA isolated from cells exposed to [•]NO,³⁸ or in tissues from a mouse model of [•]NO over-production (Pang et al, manuscript submitted for publication). To account for these observations, Glaser and coworkers recently proposed an attractive model that explains the predominance of dX and predicts that the proportion of dO will be maximal in solvent-exposed nucleoside reactions of dG and lowest in the constrained environment of double-stranded DNA under biological conditions of pH.³⁹

Several recent studies have revealed novel and unexpected features of nitrosative deamination of DNA and RNA. Having overcome critical problems with deamination artifacts caused by deaminase contamination,^{33,38} we developed LC-MS methods to define the spectrum of DNA lesions (Fig. 2) and the kinetics of their formation (Fig. 3A) in purified DNA exposed to biologically relevant concentrations of [•]NO and O₂ (steady-state concentrations of 1-2 μ M [•]NO and 150-200 μ M O₂).³³ As expected, the rates of formation of dX, dI and dU are nearly identical and, as noted earlier, dO was not produced at detectable levels. The surprising observation was the formation of abasic sites, presumably by N⁷-nitrosation of purines.

These observations of nitrosative deamination of DNA in vitro were then compared to those obtained by exposure of human lymphoblastoid TK6 cells to [•]NO and O₂ under identical conditions.³⁸ As shown in Figure 3B, the kinetics of DNA deamination were 3- to 4-fold slower in cells than in purified DNA, with significant elevations of the deamination products only when the cells experienced extensive cytotoxicity. Again, dO was not detected in cells.

Similar results were obtained by Halliwell and coworkers, who have performed one of the few well-controlled studies, in terms of deamination artifacts, of hypoxanthine formation in purified DNA and in DNA isolated from [•]NO-exposed tissues.⁴⁰ They observed background levels of dI in DNA from Jurkat cells ranging from 1-3 per 10⁶ nucleotides,⁴⁰ which compares favorably to our observation of ~5 dI per 10⁶ nucleotides in TK6 cells.³⁸ The modest increase in the steady-state levels of DNA deamination products in cells exposed to relatively cytotoxic levels of [•]NO suggest that nuclear DNA has limited exposure to nitrosating species or that high levels of formation are balanced by rapid repair of nucleobase deamination lesions in DNA. The latter seems unlikely given the lack of significant change in the levels of deamination products after a 24 hr post-exposure incubation.³⁸ In support of the limited deamination chemistry in the cellular environment, we have observed similar modest increases in nucleobase deamination in RNA isolated from [•]NO-exposed TK6 cells (Fig. 3C; Pang and Dedon, manuscript in preparation). Finally, similar observations of modest nucleobase deamination in DNA were made in rodent models of inflammation, as discussed shortly.⁴⁰

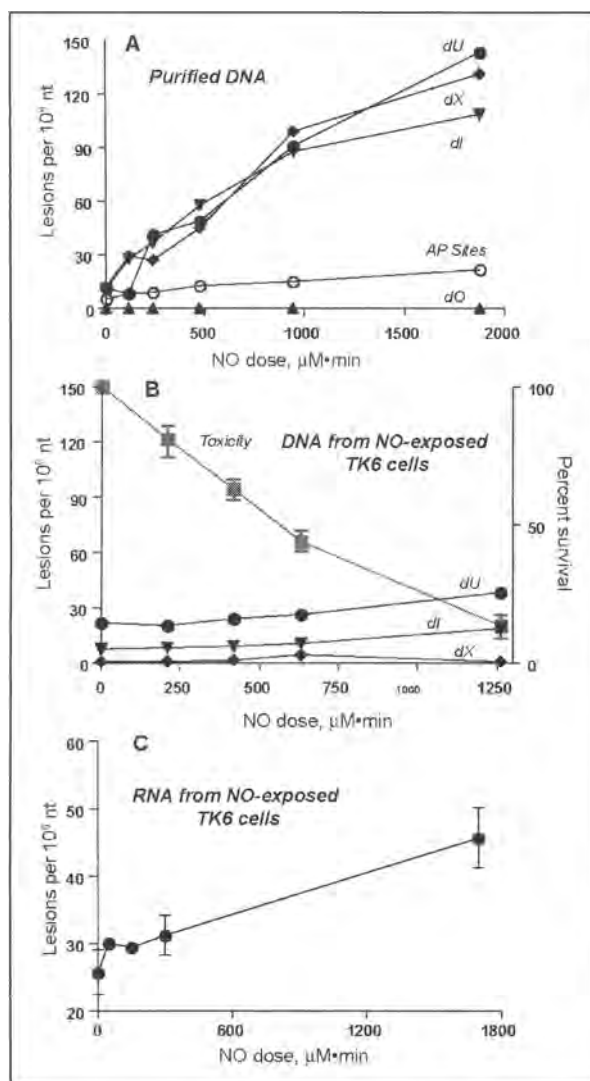


Figure 3. Kinetics of Nitrosative deamination of DNA (A,B) and RNA (C) by controlled delivery of biologically relevant steady-state levels of NO and O_2 to (A) isolated DNA and (B,C) human lymphoblastoid TK6 cells.

Oxidation of DNA by Peroxynitrite and Nitrosoperoxycarbonate

DNA is also subject to oxidation and nitration by chemical mediators of inflammation, mainly as a consequence of reactions with ONOO^- and its carbon dioxide conjugate, ONOOCO_2^- , with additional contributions from $^*\text{NO}_2$ (Fig. 1).^{41,42} While ONOO^- causes mainly deoxyribose oxidation in DNA,^{20,43} the presence of millimolar concentrations of carbon dioxide in tissues leads to the formation of ONOOCO_2^- that preferentially causes oxidation and nitration of guanine nucleobases in DNA.^{20,43,44} Given the completeness of recent reviews of guanine oxidation,⁴⁵⁻⁴⁷ the focus here will be on deoxyribose oxidation by

ONOO^- and ONOOCO_2^- and on the recent observation of the unusual sequence selectivity for the oxidation of guanine by ONOOCO_2^- .

Deoxyribose Oxidation Chemistry

Deoxyribose oxidation in DNA has been the subject of significant study with ionizing radiation,⁴⁸ Fe-EDTA⁴⁹ and a host of oxidizing agents such as enediynes,⁵⁰ porphyrins⁵¹ and others, and it results in the spectrum of products shown in Figure 4. However, few studies have addressed, in a rigorously quantitative manner, the oxidation of deoxyribose in DNA by chemical mediators of inflammation. As noted earlier, both sugar and base damage result from exposure of DNA to ONOO^- *in vitro*.^{43,52} It has recently been recognized that the proportions of the

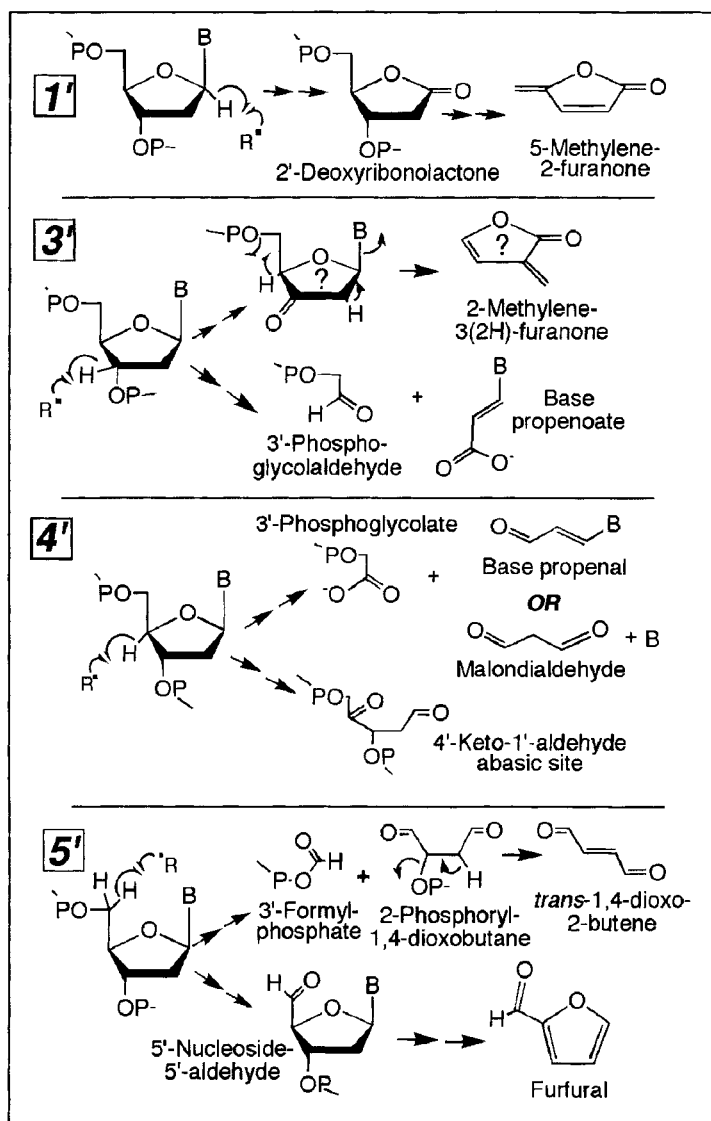


Figure 4. Chemistry of deoxyribose oxidation.

various DNA products are strongly dependent on the presence of CO_2 and thus the formation of ONOOCO_2^- .^{43,44} While ONOO^- causes predominantly deoxyribose oxidation (i.e., strand breaks and oxidized abasic sites) to the extent of 70–80% of total DNA damage,⁴³ the presence of dissolved CO_2 , such as occurs in tissues and in bicarbonate buffers equilibrated at pH 7.4, shifts the reactivity to predominantly guanine oxidation and nitration.^{43,44} Interestingly, while there is a major shift in chemistry from deoxyribose to nucleobases in the presence of CO_2 , the total quantity of DNA damage remains roughly constant.⁴³ The suppression of deoxyribose oxidation events with increasing carbon dioxide concentrations is nearly complete above 50 mM bicarbonate⁴⁴ (Collins and Dedon, unpublished observations). However, deoxyribose oxidation represents about 20% of total DNA damage at carbon dioxide concentrations of 1–2 mM that occur physiologically and in solutions of 20–30 mM bicarbonate;^{43,44} the normal sum of carbonate and bicarbonate in human blood is ~25 mM.⁵³ Thus, there is reason to expect that the spectrum of oxidative DNA damage under physiological conditions will represent a mixture of chemistries arising from both ONOO^- and ONOOCO_2^- . This was apparent in recent studies of base propenal-derived M_1dG formation in cells exposed to ONOO^- , in which deoxyribose oxidation produced base propenal that led to the formation of the DNA adduct.⁵⁴ Further details about the chemistry of deoxyribose oxidation will be considered in later sections of this chapter.

Nucleobase Oxidation Chemistry

As has been thoroughly addressed in recent reviews,^{45–47} guanine represents the major nucleobase target for oxidation by chemical mediators of inflammation as a result of its low oxidation potential relative to other nucleobases ($E^\circ = 1.29 \text{ V}$ vs. NHE^{55}). The major products arising from direct reactions of dG in DNA with ONOO^- and ONOOCO_2^- are shown in Figure 5 and include 8-nitro-2'-deoxyguanosine (8-nitrodG), which rapidly depurinates to 8-nitroG and an abasic site; 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG); 5-guanidino-4-nitroimidazole (NitroIm); and 2,2-diamino-4-[(2-deoxy- β -D-erythro-pentofuranosyl)amino]-5(2H)-oxazolone (oxazolone; Oz). Of some importance to understanding the determinants of the spectrum of DNA lesions at sites of inflammation is the fact that 8-oxodG, a major product of dG oxidation, is at least 1000-fold more reactive than the parent dG⁵⁶ toward further oxidation ($E^\circ = 0.74 \text{ V}$ vs. NHE^{57}) and its oxidation gives rise to a host of more stable products (Fig. 5). Of these products, only the spiroaminodihydantoin lesion has been amenable to detection in cells due to the limited sensitivities of current analytical methods (~1 lesion per 10^7 nucleotides).⁵⁸ This problem may well reflect the fact that we all measure steady-state levels of DNA lesions, in which the balance of formation and repair may lead to undetectably low levels of some DNA lesions.

One factor complicating our understanding of guanine oxidation in DNA is the concept of charge transfer. Determinants of the location and quantity of mutagenic oxidative DNA damage have been extensively studied in recent years for a variety of one-electron oxidants, including photo-activated Rh(III)-phenanthrolines,⁵⁹ anthraquinones,⁶⁰ naphthalimides,⁶¹ and riboflavin,⁶² as well as metal-based systems⁶³ and lasers.⁶⁴ All of these agents selectively oxidize the 5'-guanine in a run of guanines, such as GG and GGG. This behavior has been attributed to the migration of the initial radical cation to a low-energy site, with a termination of the charge migration at guanines with the lowest ionization potential by trapping and subsequent product formation.^{59,65–67} In support of this model, Saito et al observed a pronounced inverse correlation between the calculated ionization potentials for guanines in different sequence contexts^{68,69} and the reactivity of those guanines toward riboflavin-mediated photo-oxidation.⁶² This model led to the general conclusion that DNA oxidizing agents will produce high levels of damage in the genome at the most readily oxidized guanines (GG, GGG) either directly or as a result of charge migration to the lowest energy hole trap. At the simplest level this is consistent with the predominance of damage at dG, the nucleobase with the lowest oxidation potential of the four canonical nucleobases, and with the observed preferential formation of oxidative damage at 8-oxodG.⁶⁷

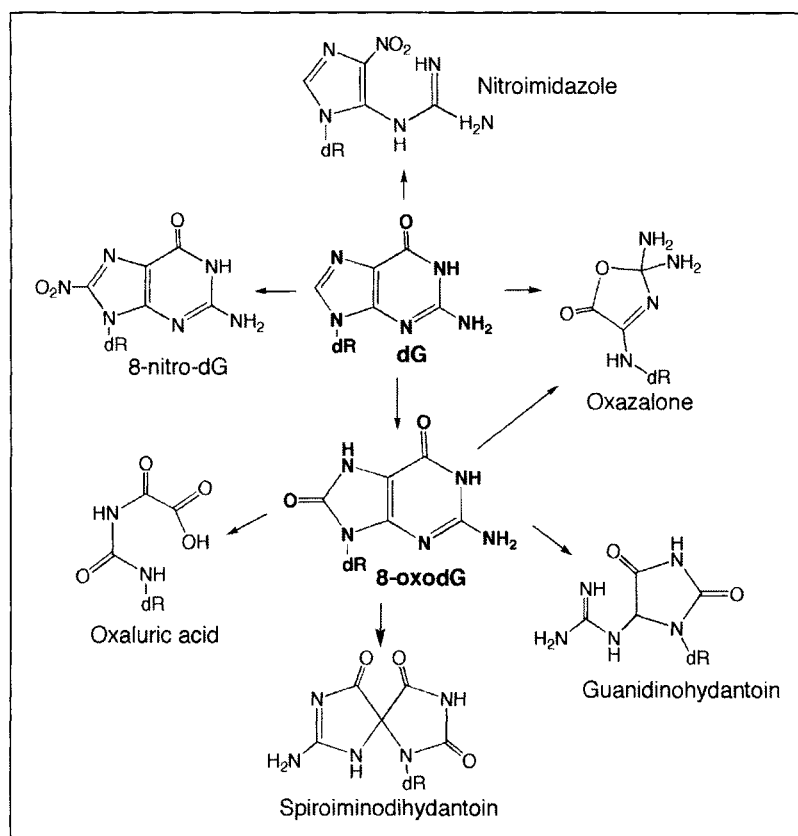


Figure 5. Chemistry of guanine oxidation by chemical mediators of inflammation.

Recent observations, however, with ONOOCO_2^- -induced DNA damage indicate that this model may not account for the location of DNA damage in the human genome.⁷⁰ Of the oxidants $\text{CO}_3^{\cdot-}$ and NO_2 that arise from homolytic bond scission of ONOOCO_2^- (Fig. 1), the oxidation potential of NO_2 is not sufficient to oxidize guanine in DNA,⁷¹ while $\text{CO}_3^{\cdot-}$ selectively reacts with guanine by a one-electron transfer step.⁷² Toward defining the link between inflammation and cancer,²⁰ we used the pSP189 shuttle vector to examine the relationship between the DNA damage and the mutations caused by ONOOCO_2^- .⁴³ There was a clear correlation between DNA damage and mutations,⁴³ but reexamination of those data revealed a striking and unexpected selectivity of ONOOCO_2^- for guanine bases in TGC and AGC sequence contexts. This qualitative observation was then quantified by comparing the reactivity of ONOOCO_2^- with guanine bases located at the center of all possible trinucleotide sequence contexts using the series of double-stranded oligodeoxynucleotides employed by Saito and coworkers in their studies of riboflavin-mediated photooxidation.⁶² These oligonucleotides contained two variable guanine contexts and an invariant TGG triplet that served as an internal control for the normalization of damage at the other sites. Nucleobase damage produced by ONOOCO_2^- at each target guanine was quantified following conversion of guanine base lesions to strand breaks by treatment with either hot piperidine or formamidopyrimidine DNA glycosylase.

The general features of the traditional charge transfer model are illustrated in the plot of ionization potential versus cleavage frequency for riboflavin-mediated photooxidation shown

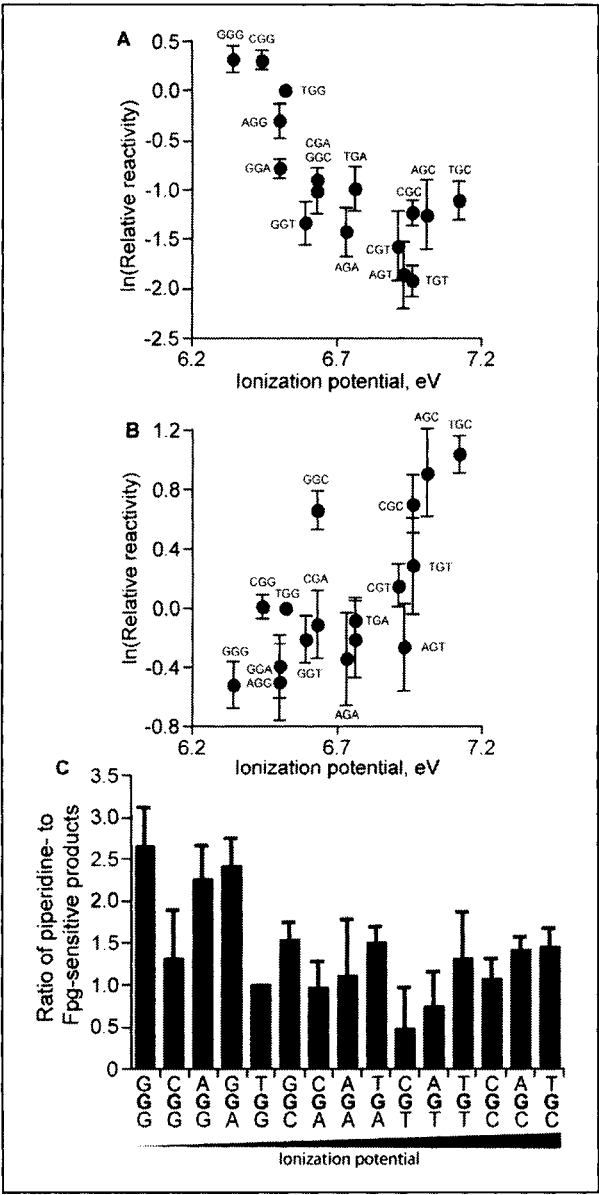


Figure 6. Paradoxical sequence selectivity for the oxidation of guanine by chemical mediators of inflammation. A) Plot of the relative reactivity of guanines in different sequence contexts toward riboflavin-mediated photooxidation as a function of the calculated ionization potential of the guanine. B) Plot of the relative reactivity of guanines in different sequence contexts toward ONOOCO_2 -induced oxidation as a function of the calculated ionization potential of the guanine. C) Sequence dependence of guanine oxidation chemistry.

in Figure 6A.^{62,68,70} There is a clear, inverse relationship between the “oxidizability” of a guanine and the frequency of damage formation at the guanine when the DNA is oxidized by riboflavin. With ONOOCO_2^- (Fig. 6B), however, the relationship is essentially inverted, with

the highest frequency of damage occurring in the AGC, TGC and CGC motifs that impart the highest ionization potential to guanine and the lowest frequency of damage occurring in the GGG sequence in which the guanines are the most easily oxidized.⁷⁰ The GGC sequence is unique in that it displays high relative reactivity with ONOOCO_2^- despite containing a GG pair that is relatively unreactive in all other sequence contexts. That the sequence selectivity of ONOOCO_2^- depends on B-DNA structure is supported by disappearance of discernable sequence-related trends in single-stranded oligodeoxynucleotides damaged by either riboflavin or ONOOCO_2^- .⁷⁰ These results persist in genomic DNA treated with ONOOCO_2^- ⁷⁰ and point to the GC motif as a major determinant of ONOOCO_2^- oxidation hotspots in DNA. The unusual sequence selectivity of ONOOCO_2^- for oxidation of guanine nucleobases appears to be shared by other biologically relevant oxidants⁷³ and suggests that current models of guanine oxidation and charge transfer in DNA could follow paradigms other than those proposed in the generally accepted models.^{59,62,65-67}

Another interesting feature of the sequence dependence of guanine oxidation is the observed variation in oxidation chemistry as a function of sequence context.^{64,70} As shown in Figure 6C, the ratio of piperidine-sensitive to Fpg-sensitive damage products in several guanine sequence contexts was observed to vary over a 6- to 7-fold range. The reactivities of guanine oxidation products with piperidine and Fpg are highly variable:⁶⁴ 8-oxoG, Fapy-dG and nitroimidazole products are reactive with Fpg and resistant to piperidine;^{64,74} 8-nitroG and 2,2-diaminooxazolone (dZ) are resistant to Fpg and reactive with piperidine;^{43,64,75} and spiroiminodihydantoin is equally susceptible to Fpg and piperidine.⁷⁰ Thus, the differences in reactions with Fpg and piperidine depicted in Figure 6C clearly indicate that formation of the different guanine oxidation products depends on sequence context, which has implications for predicting the mutational spectra arising in cells subjected to inflammation and other oxidative stresses.

Reactions of DNA with Endogenous Electrophiles Arising from Oxidation

The reactive oxygen and nitrogen species arising during inflammation and oxidative stresses in general can react with cellular molecules other than DNA, including polyunsaturated fatty acids,⁷⁶⁻⁷⁸ carbohydrates^{79,80} and proteins.⁸¹⁻⁸³ The oxidation and peroxidation of these molecules generates a variety of electrophilic species capable of reacting with DNA and RNA bases to form adducts. Included in this class of "indirect" DNA damage are the etheno adducts (substituted^{84,85} and unsubstituted^{78,86-88}), propano and ethano adducts,⁸⁹⁻⁹³ glyoxal adducts,^{94,95} and a more recently discovered series of DNA adducts derived from deoxyribose oxidation products.^{54,96-100} The latter will be addressed in a separate section of this chapter. Given the thorough review of these DNA lesions (see above references), only the recent advances in lipid peroxidation-derived etheno adducts will be covered here.

Lipid peroxidation induces the formation of a variety of α,β -unsaturated aldehydes such as trans-4-hydroxy-2-nonenal, acrolein and 4-oxo-2-nonenal.^{76,78} These electrophiles can react with DNA bases to form, among other adducts, substituted and unsubstituted etheno adducts, such as the 1,N⁶-etheno-2'-deoxyadenosine (ϵ dA), 3,N⁴-etheno-2'-deoxycytidine (ϵ dC), and 1,N²-etheno-2'-deoxyguanosine (1,N²- ϵ dG) adducts shown in Figure 7.^{84,87,88} Blair and coworkers have used a targeted mass spectrometric approach to quantify three varieties of etheno-type adducts (Fig. 7) derived mainly from products of peroxidation of linoleic acid,¹⁰¹ the simplest and most abundant polyunsaturated fatty acid of mammalian cell membranes.¹⁰² As shown in Figure 7, peroxidation of linoleate or arachidonate can be initiated by many different oxidizing agents as well as by cyclooxygenases,¹⁰¹ with the resulting α,β -unsaturated aldehydes giving rise to the well known unsubstituted etheno adducts of dC, dA and dG in DNA,⁸⁷ as well as alkyl-substituted etheno adducts^{103,104} and protein adducts.¹⁰⁵ Blair and coworkers quantified the carboxynonanone-, heptanone- and unsubstituted etheno adducts of dG, dA and dC (Fig. 7) in wild-type mice and the "Min" mouse model for intestinal neoplasia.⁸⁴

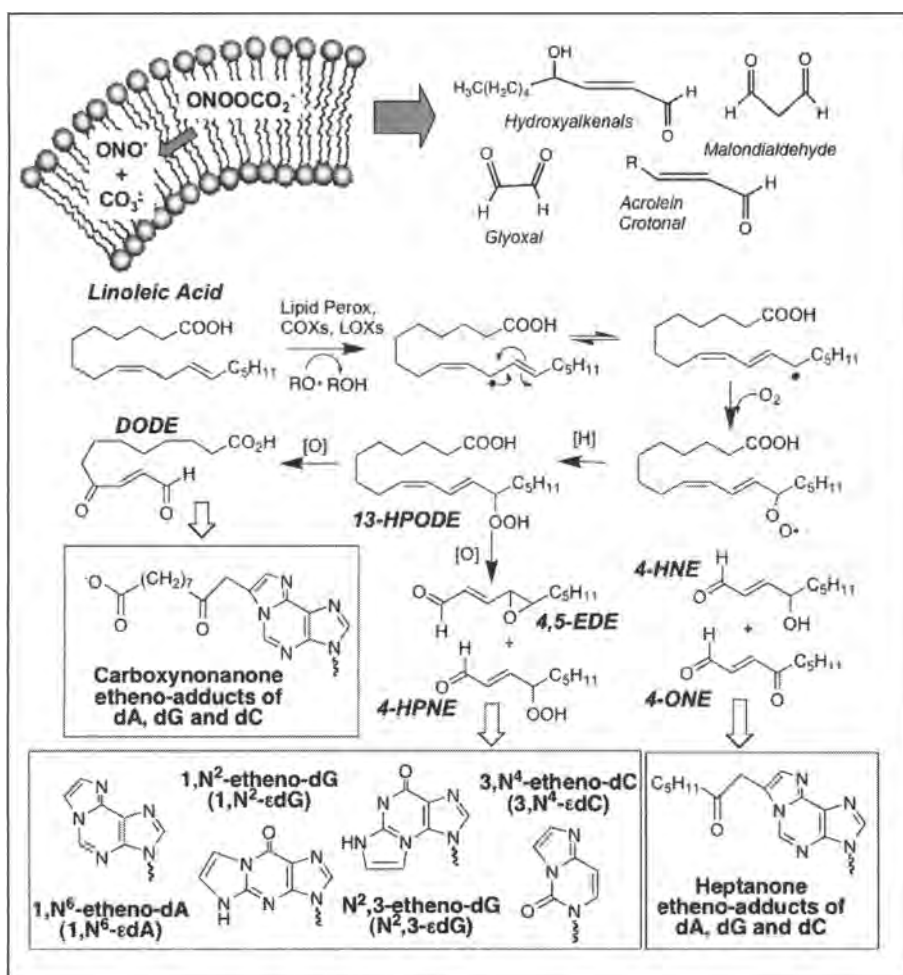


Figure 7. Chemistry of lipid peroxidation.

with the latter containing up-regulated COX-2 presumably leading to increased lipid peroxidation. The heptanone-etheno adducts of dC and dG (Fig. 7) were increased 3- to 10-fold in the Min mice, while the carboxynonanone adducts and the unsubstituted etheno adducts were not apparent above a putative detection limit of 1 per 10^8 nt in their LC-MS/MS assay. These studies expand the repertoire of lipid peroxidation-derived DNA adducts and provide a starting point for understanding the role of lipid peroxidation in the cellular burden of toxic DNA damage.

Oxidative DNA Damage as a Source of DNA Adduct-Forming Electrophiles

Relative to direct damage to DNA bases, such as the formation of 8-oxodG, the genetic toxicology of the oxidation of deoxyribose in DNA has only recently become the focus of significant study. Deoxyribose oxidation results in the formation of oxidized abasic sites, strand breaks terminated with a variety of sugar residues, and freely diffusible degradation

products, as illustrated in Figure 4. For example, 4'-oxidation by DNA-cleaving antibiotics^{48,50,106} partitions along two pathways to form a 3'-phosphoglycolate residue with release of base propenal; or a 2-deoxypentose-4-ulose abasic site.⁵⁰ However, recent studies performed by von Sonntag and our group have revealed an alternative 4'-chemistry arising with Fe^{+2} -EDTA⁵⁴ and γ -radiation^{54,107} that results in malondialdehyde and a free base rather than a base propenal (Fig. 4). Regarding the other sugar positions, 5'-oxidation results in two sets of products, including a 3'-formylphosphate residue¹⁰⁸ and, as we have discovered,¹⁰⁹ a 2-phosphoryl-1, 4-dioxobutane species; or a nucleoside-5'-aldehyde that undergoes β -elimination to form furfural.¹¹⁰ The 3'-oxidation pathway results in a 3'-phosphoglycolaldehyde-ended fragment with release of base propenoate;^{97,111-113} or possibly a fragment possessing a nucleoside-3'-ketone residue that should undergo β -elimination to release 2-methylene-3(2H)-furanone.¹¹⁴ Finally, 1'-oxidation by many agents¹¹⁵⁻¹¹⁷ yields a 2'-deoxyribonolactone abasic site that undergoes β -elimination to form a methylene furanone species. There is strong evidence for covalent cross-linking between DNA repair proteins and the abasic site,^{118,119} and a recent report points to 1'-oxidation as the dominant form of deoxyribose oxidation caused by ionizing radiation.¹²⁰

Many of these deoxyribose oxidation products are electrophilic and capable of reacting with local nucleophiles to form both DNA and protein adducts. This is illustrated by our observation that base propenals arising from 4'-oxidation of deoxyribose undergo a facile reaction with dG to form the pyrimidopurinone adduct of dG, M_1dG , in isolated DNA^{96,99} and in cells,⁵⁴ a behavior explained by the chemical similarities of malondialdehyde and base propenals (Fig. 8A). Our recent studies in *E. coli* revealed that base propenals, and not malondialdehyde, represent the major source of M_1dG in biological systems.⁵⁴ This is consistent with the studies of Kadlubar et al.,¹²¹ who observed that the level of M_1G correlated with 8-oxodG, but not with lipid peroxidation-derived etheno adducts. All of these results suggest that M_1G is not a biomarker for the genetic toxicology of lipid peroxidation.

Another example involves the phosphoglycolaldehyde product of 3'-oxidation of deoxyribose, which leads to formation of glyoxal adducts in DNA.⁹⁷ Glyoxal is a ubiquitous dicarbonyl oxidation product of lipids and carbohydrates, and it represents one of the advanced glycation end products involved in the pathology of diabetes and aging.^{122,123} Glyoxal reacts with guanine bases in DNA to form 1, N^2 -adducts, the two diastereomers of 3-(2-deoxy- β -D-erythropento-furansyl)-6,7-dihydro-6,7-dihydroxyimidazo-[1,2-a]purine-9(3H)one (Fig. 8B).¹²⁴⁻¹²⁶ The repertoire of glyoxal sources was extended by Murata-Kamiya et al with their demonstration that glyoxal and its 1, N^2 -adducts of dG are generated in DNA oxidized by Fe^{+2} -EDTA.¹²⁷ We have shown that 3'-phosphoglycolaldehyde residues in oxidized DNA represent the source of the glyoxal.⁹⁷ This deoxyribose oxidation product undergoes an oxygen- and radical-independent oxidation event to form glyoxal and subsequently the glyoxal adducts of dG.⁹⁷

The Peterson group and our group recently reported that DNA oxidation leads to the formation of oxadiazabicyclo(3.3.0)octamine adducts of dC and dA consistent with generation of electrophilic products of 5'-oxidation of deoxyribose in DNA.¹⁰⁹ As shown in Figure 4, the chemistry of 5'-oxidation of deoxyribose in DNA by a variety of DNA-cleaving agents, including enediynes⁵⁰ and manganese porphyrins,⁵¹ partitions to form strand breaks containing 3'-phosphate and 5'-nucleoside-5'-aldehyde residues, or a 3'-formylphosphate species accompanied by a four-carbon fragment that we and others have identified as a 5'-(2-phosphoryl-1,4-dioxobutane) residue.^{109,128} We recently showed that this dialdehydic residue undergoes β -elimination to form trans-1,4-dioxo-2-butene,¹⁰⁹ a highly reactive α,β -unsaturated dicarbonyl species. Further, the cis-isomer of 1,4-dioxo-2-butene is a metabolite of furan,¹²⁹ an industrial solvent and component of food, smog and cigarette smoke^{130,131} that has been shown to be carcinogenic in rodents.^{130,131} We have demonstrated that both of these electrophilic isomers react with bases in DNA in vitro to form novel, stable oxadiazabicyclo(3.3.0)octamine adducts of dC, dG and dA under biologically

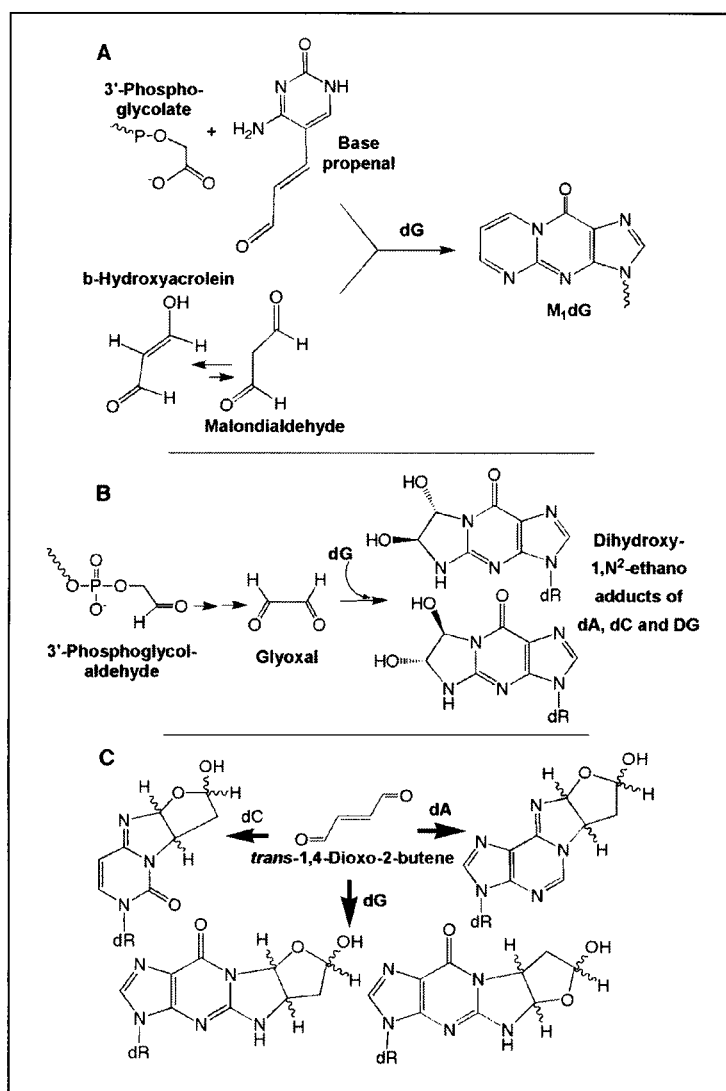


Figure 8. DNA adducts arising from products of deoxyribose oxidation in DNA.

relevant conditions (Fig. 8).^{98,100,132} The dC and dA adducts were also detected in DNA from bacteria exposed to mutagenic concentrations of cis-1,4-dioxo-2-butene, indicating that these adducts are likely to be mutagenic.^{133,134}

Putting It All Together: DNA Biomarkers of Inflammation in Vivo

With implications for biomarker development, one of the key questions involving inflammation-related chemical biology is whether there is a predominant chemistry affecting DNA at sites of inflammation, such as direct nitrosative or oxidative DNA damage by chemical mediators of inflammation, or indirect damage in the form of adducts derived from inflammation-induced electrophiles. As noted earlier, there have been numerous studies in

which individual DNA lesions or lesion classes have been studied in both rodent and human tissues affected by oxidative stress or inflammation. For example, Bartsch and coworkers quantified the etheno adducts in the SJL mouse model of *NO over-production,¹³⁵ in rats and humans suffering from the copper over-load of Wilson's disease,⁷⁸ and in humans with chronic hepatitis.⁷⁸ These are excellent studies from the perspective of assessing a single biomarker, such as the etheno adducts. However, they do not provide a thorough assessment of the spectrum of chemistries of potential importance in both biomarker development and in understanding the mechanisms relating inflammation to disease.

This problem has been approached by quantification of a battery of potential DNA biomarkers of inflammation in the SJL mouse model of inflammation.¹³⁶⁻¹³⁸ This model entails large increases in macrophage-derived *NO production in spleen, lymph nodes and liver following injection of superantigen-bearing, reticulum cell sarcoma-derived RcsX cells that leads to widespread macrophage activation. The increased production of *NO is associated with increases in mutation frequency¹³⁷ and in tissue levels of nitrotyrosine¹³⁹ and the etheno adducts ϵ -dA and ϵ -dC.¹³⁵ We used this model to quantify a battery of DNA adducts thought to reflect the chemistries arising at sites of inflammation: deamination products dU, dX, dO and dI (Fig. 2); oxidation products M₁dG (Fig. 8), 8-oxodG, spiroiminodihydantoin (Sp), guanidinohydantoin (Gh), NitroIm and oxazolone (Ox) (Fig. 5); and the etheno adducts of dA and dG (Fig. 7). The results revealed substantial increases in only the etheno adducts (3- to 4-fold) with insignificant changes in all of the other lesion chemistries (Pang et al, manuscript submitted for publication). The results with the etheno adducts are consistent with the observations of Bartsch and coworkers,¹³⁵ while the absence of inflammation-induced changes in 8-oxodG have also been observed by Gal et al in the SJL mouse¹³⁹ and by Kadlubar et al in human pancreatitis.¹²¹ Similarly, Halliwell and coworkers did not observe changes in the level of dI in a rat model of inflammation.⁴⁰

There are several possible explanations for the minimal increases in the levels of the oxidative and nitrosative DNA lesions. One involves DNA repair as discussed earlier. Another possible explanation is a lesion dilution effect. The basis for this effect lies in the analysis of DNA extracted from the variety of native and invading cell types that comprise an inflamed organ such as the spleen. Only a small fraction of the cells present in the spleen or other organs may be sustaining the bulk of the DNA damage and the adduct load is diluted by undamaged DNA from unaffected cells. Finally, it is possible that reactive nitrogen and oxygen species arising from the macrophage-derived *NO are unable to access the nucleus or are consumed before reacting with nuclear DNA, thus obviating direct reactions of N_2O_3 , *NO_2 or $ONOOO_2^-$ with DNA.

Clearly, lipid peroxidation and the lipid environment play key roles in inflammation-related pathology. The importance of lipid chemistry in inflammation is substantiated by numerous observations of significantly elevated etheno adducts in DNA in a variety of clinical disorders,⁷⁸ including Wilson's disease^{140,141} and colonic polyps of familial adenomatous polyposis patients.⁸⁶ Of particular interest is the observation that the levels of etheno adducts were increased in the kidney of SJL mice (Pang et al, manuscript submitted for publication), which is not an organ subject to the massive infiltration of macrophages and neutrophils observed in the spleen in the RcsX-treated mice (i.e., not a target organ).^{136,137,139} These results suggest that nontarget organs may experience similar oxidative and nitrosative stresses as target organs but "at a distance," perhaps as a consequence of cellular reactions to blood-borne species such as cytokines, nitrosoglutathione, redox active metals released from damaged cells, and other relatively stable chemical mediators of inflammation.

Acknowledgements

This work was supported by grants from the National Institutes of Health (CA026735, GM059790, CA103146, CA110261, CA116318 and ES002109).

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CHAPTER 16

The Role of Antioxidants in the Prevention of Oxidative Damage to Nucleic Acids

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Abstract

It is commonly assumed that ingestion of antioxidants is associated with low levels of oxidatively damaged DNA although this is far from conclusive in human intervention trials. A collective interpretation is difficult because many studies lack sufficient control and have unrealistically high baseline levels of oxidative DNA damage in human white blood cells (WBC). A survey of studies on the antioxidant hypothesis in terms of oxidative DNA damage excretion products in urine indicates that ingestion of antioxidant-rich foods may be more effective than single antioxidants. In WBC, there is some evidence of beneficial effects of ingestion of antioxidants and antioxidant-rich foods. This suggests intake of antioxidants either in tablet form or as natural ingredients of foods are associated with beneficial effects on oxidative stress status, but the effect is smaller than previously expected and supplementation of antioxidants to healthy and sufficiently nourished individuals may not be of large public health relevance.

Introduction

The role of antioxidants in the prevention of DNA oxidation can be investigated in a wide array of experimental settings, ranging from cell free systems, to large intervention studies. 'Antioxidant' is a widely used term that is difficult to define clearly in biological systems and the effect may depend on the experimental setting. We use the term antioxidant as a broad definition of any substance that can prevent oxidation of biomolecules, either directly by scavenging ROS, or indirectly by upregulating the antioxidant defense or DNA repair systems. The indirect antioxidant effect may be induced by xenobiotics or components in vegetables that are not scavengers or even considered potentially harmful e.g., isothiocyanates from cruciferous vegetables. The types of antioxidants can roughly be grouped into categories in relation to administration, as follows: (1) single antioxidants; (2) multiple antioxidants; (3) extracts or juices of natural food products. Antioxidants like vitamin C, vitamin E, carotenoids, and flavonoids have been identified in a large range of natural food products,¹ but fruit and vegetables also contain a mixture of other antioxidants and bioactive substances that are less well investigated in terms of antioxidant properties.

Cell free systems and cell cultures exposed to a ROS generating system are commonly used to investigate the scavenging effect of dietary compounds. The large number of investigations addressing the in vitro antioxidant potential of phytochemicals by far impedes a thorough

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discussion. Both in vitro experimental settings have the advantage of being fast and reliable, but extrapolations to the effect in humans are often difficult, because the concentrations of antioxidants are unrealistic and the ROS-generating system can be disproportionate compared to the balance between antioxidants and ROS that exists in vivo. A further caveat is that antioxidants in high concentrations act as pro-oxidants; this in vitro effect is well known for vitamin C² but also other phytochemicals show the same pattern e.g., quercetin.^{3,4} Besides establishing the scavenging ability of antioxidants, cell culture experiments can gain information about the regulation of gene expression of antioxidant proteins or DNA repair enzymes. Genetically altered cell cultures or gene silencing by small interfering RNA may be suitable model systems for mechanistic studies.

Animal experimental models offer several opportunities related to the understanding of the mechanism of antioxidants and this consequently strengthens the biological plausibility. An obvious advantage is the possibility of investigating the effect in the presumed target tissues such as colon and lung. More importantly, it is possible to test chemoprevention as interactions between antioxidants and known dietary genotoxic components, e.g., dietary supplementation with β -carotene suppressed generation of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-OHdG) by diesel exhaust particles in the lung of mice.⁵ Several rodent experimental models have shown that tea polyphenols inhibit carcinogen-induced DNA oxidation in various organs, whereas the effect on spontaneous oxidized DNA bases is limited.⁶ However, one should be aware that rodents differ in their requirement for antioxidants, which may hamper the extrapolation to humans. As an example, mice and rats synthesize ascorbate and α -tocopherol *de novo*, whereas humans and Guinea pigs lack this ability and require dietary vitamins C and E. Interestingly, using Guinea pigs as a model revealed no effect on hepatic 8-OHdG by variations in dietary vitamin C or E supplementation.⁷ This suggests that the pro-oxidant effect of vitamin C shown in vitro is difficult to reproduce in rodents or humans. It should also be stressed that the dose of antioxidants is crucial and that high-dose administrations of antioxidants may be toxic in some rodent organs, e.g., ingestion of Brussels sprouts extract in high doses diminishes 8-OHdG induced by 2-nitropropane in rat liver,⁸ but increases the endogenous level of 8-OHdG in the same organ.⁹ In fact, it is common that antioxidants in animal experimental models surprisingly reveal toxic rather than chemopreventive effects, e.g., higher levels of DNA oxidation have been observed in rat lymphocytes following supplementation with lycopene, quercetin, and resveratrol to the animals.¹⁰

Intuitively, the most relevant way of exploring antioxidant effects in humans are supplementation trials, but measurements of oxidatively damaged DNA are often restricted to surrogate tissues such as white blood cells (WBC) and urine. The WBC are usually mononuclear blood cells encompassing lymphocytes and monocytes although some studies use total leukocytes. With the ability to detect 8-OHdG, the first of many antioxidant supplementation trials with focus on this lesion in WBC appeared at the beginning of the 1990s.¹¹ At the same time, reliable detection of 8-OHdG in urine became possible,¹² and this was soon followed by antioxidant trials with urinary 8-OHdG excretion as a key biomarker. However, by far the most popular assay in antioxidant intervention trials has been the comet assay, detecting DNA strand breaks (SB) or, the enzyme-modified version of the comet assay, that detects oxidized purines (including 8-OHdG) and pyrimidines by formamidopyrimidine DNA glycosylase (FPG) and endonuclease III (ENDOIII), respectively. Also *ex vivo* exposure of cells to DNA strand breaking agents such as H₂O₂ or ionizing radiation has been used as a semiquantitative measurement of the donor's antioxidative status. This modification of the comet assay is based on the notion that the intracellular content of antioxidants will affect the DNA breakage.

A brief survey of databases, like PubMed, reveals that more than 100 intervention studies have investigated the effect of antioxidants in human WBC and urine. Unfortunately, the results generated by many of the studies can be challenged because of poor design quality and

unrealistic levels of DNA oxidation. We have described that about half of the antioxidant intervention studies were poorly designed with no placebo group included.^{13,14} Furthermore, as consequence of the emergent focus on the baseline level of DNA oxidation, it has been argued that the level of DNA damage should be a major consideration in interpretations of the conclusions of antioxidant intervention studies.¹⁵ A thorough overview is feasible only on studies including data measured by either the comet assay (or related assays) or 8-OHdG/8-OHGua, because other types of oxidative lesion have been examined in very few studies and potential problems related to the methodology for some lesions limit the interpretation of results, e.g., oxidative adenine lesions.

Evaluation of Antioxidant Intervention Studies

Ideally, antioxidant intervention studies with biomarkers of DNA damage as the endpoint should be evaluated by meta-analysis, because it allows stronger conclusions by combining underpowered studies. The heterogeneity of the antioxidant studies with respect to type, dose and duration of intervention, design, population, type of biomarkers etc. precludes that possibility. Instead, we use a set of evaluation criteria as follows:

1. The duration of the intervention and washout periods.
2. The type of lesion; DNA strand breaks (SB), ENDOIII sites, 8-OHGua/8-OHdG, and FPG sites.
3. The type of antioxidants categorized as single antioxidants (e.g., vitamin C), multiple antioxidants (e.g., combinations of vitamin C and vitamin E, or multi-vitamin tablets), and antioxidant-rich foods (e.g., fruit juices or Brussels sprouts).
4. Inclusion of subjects who smoke. A population of smokers may be regarded as oxidatively stressed and could have better benefit of antioxidant administration. This could be because smokers compared to nonsmokers have a faster turn-over of vitamin C and thus require a higher habitual dietary vitamin C intake to maintain plasma level.¹⁶
5. The power of the study. An evaluation of the study power is difficult because few papers provide an a priori power calculation or have sufficient data to calculate the power. The number of subjects in the study can be used as a crude indicator of the statistical power. A more reliable proxy measure of the power is obtained by comparing the actual number of subjects in the study and number of subjects required to detect a certain difference between the groups of the study based on the mean and standard deviation of the baseline values of oxidative DNA damage. We have used this assessment by calculating the number of subjects required to detect a 50% difference and assuming a statistical level of 5% (α -value) and accepting a 20% chance of committing a type II statistical error (β -value).
6. The design of the study. In two investigations we have documented that the study design was of major importance for the outcome of long-term intervention trials.^{13,14} There was a clear association between poorly controlled studies and beneficial effects. The reason for this association could be straightforward; period effects (i.e., changes in the level of biomarkers over time that are unrelated to the intervention) are misinterpreted as treatment effects in poorly controlled studies because placebo treatments are lacking. We have devised a simple scoring system where a study can obtain a score from zero (weak design) to three (strong design): points are given for studies with a placebo group, parallel design, and inclusion of sampling after the intervention period.¹³ The scoring system does not discriminate between crossover and placebo-controlled parallel intervention studies (i.e., score 2), whereas these designs have higher score compared with sequential studies (i.e., score 0). The point given to studies that include samplings after the end of the intervention period is related to causality, i.e., the effect on the biomarker should be reversed when the intervention period is completed.

7. The level of oxidative DNA damage at baseline. We have set as a criterion that the level of oxidative DNA damage should be less than 10 8-OHdG per 10^6 dG in WBC. Detection of 8-OHdG in the urine is mainly measured by HPLC and ELISA assays. Early assessments indicated a poor correlation between these measurements.¹⁷ More recent achievements and better isolation techniques for 8-OHdG in urine have yielded higher correlations.^{18,19} However, in a historical perspective many of the studies used suboptimal ELISA methods. In addition, there is difference in the values obtained by ELISA and HPLC measurements; the former produces at least 2-fold higher excretion rates than the latter.^{18,19} It is possible that the antibody-based detection of 8-OHdG is associated with nonspecific detection of structurally related derivatives of 8-OHdGua. It is quite common that the urine collection period differs between studies. The most preferred periods of collection are spot urine and 24 h samples, but collection periods in between may also be chosen. Spot urine samples are commonly chosen because they are convenient for the subjects and therefore favor compliance. Correlations of 8-OHdG measurements detected by spot and 24 h urine samples have been reported to be 0.87 and 0.50 in samples detected respectively by ELISA²⁰ and HPLC.²¹ The collection period appears less important for the antibody-based detection of 8-OHdG compared to the HPLC measurements. Probably this does not indicate that the ELISA method is more reliable than HPLC measurements. Rather more likely is the explanation that components in urine, which cross-react with the antibody, are excreted at higher level or more stable in various urine collection periods.
8. The validity of the intervention trials can be assessed as the change in plasma or urine concentration of antioxidants. Both compliance and bioavailability of the supplements will affect the validity of the supplementation. Significant alteration in the concentration of at least one plasma or urine antioxidant can be regarded as a successful intervention. Some investigations measure the antioxidant capacity by, e.g., the Trolox-equivalent and ferric-reducing ability of plasma (FRAP) assays, rather than plasma antioxidants. We do not consider these assays valid for assessment of compliance or effectiveness of the supplementation because they measure types of antioxidants of endogenous origin in addition to those derived from the supplement.

Effect of Antioxidant Supplementation on Oxidative DNA Damage in WBC

In our literature surveys, we find that only about half ($n = 22$) of the antioxidant intervention studies have the required quality to be eligible in a critical analysis based on the above criteria.^{13,14} The easiest categorisation of these studies are as single or multiple dose studies on either healthy or oxidatively-stressed subjects:

Single Dose Studies

A study among male subjects with low baseline vitamin C concentration showed a marked decrease in the level of ENDOIII and FPG sites in the first few hours after ingestion.²² On the other hand, drinking kiwifruit juice (equivalent to about 8 fruits) had no effect on the ENDOIII and FPG sites even though the vitamin C concentration in plasma increased more than 2-fold in the hours after ingestion.²³ It can be speculated that the lack of effect in the kiwifruit juice study covers an altered balance between the antioxidant effect and a pro-oxidant/adverse effect of the bolus ingestion. There are infrequently speculations of pro-oxidant effects of vitamin C or antioxidant-rich foods in human intervention studies, but it should be noted that delineation of such adverse effects is difficult because it requires a large range of doses.

Repeated Dose Studies

Repeated administrations of antioxidants usually encompass an intervention period lasting for days to months. This poses a challenge to the stability of the biomarkers and compliance of subjects. Analysis of SB in WBC is an ineffective intermediate endpoint for showing antioxidant effect although it may be relevant for overall genotoxic exposures as observed in e.g., investigations of occupational exposures.^{13,14,24} Table 1 summarizes the studies of repeated antioxidant supplementation that have investigated the impact upon ENDOIII, FPG, and 8-OHG/8-OHdG; these included 6 studies that showed protective effect^{22,25-29} and 12 studies reporting null effect.^{20,30-40}

Single Supplements

Vitamin C supplementation (80-400 mg/day for 15 wk) to healthy subjects with a high baseline level of this antioxidant in WBC was not associated with a lower level of 8-OHdG.³⁰ One study investigated the effect of vitamin E (5-7 or 80 mg/day) in combination with poly-unsaturated fatty acids (PUFA) provided as 5% or 15% of the food energy. This study showed that the content of PUFA had no effect on ENDOIII sites in subjects supplemented with 80 mg vitamin E/day for 4 wk. The subjects supplemented with 5-7 mg vitamin E/day had decreased ENDOIII sites on a diet with 5% PUFA, whereas the same endpoints increased from baseline by eating 15% PUFA.²⁶ This apparent pro-oxidant property of PUFA was not found in a study of linoleic acid supplementation,⁴¹ although this may be due to a relatively high baseline level (25 8-OHdG/10⁶ dG) of DNA oxidation that may have prevented detection of a small change in the level of DNA damage. Lower levels of 8-OHdG were observed among young subjects, but not in old subjects after ingestion of 100 mg vitamin E/day for 12 wk.²⁵ Two studies did not reveal an effect of vitamin E supplementation in terms of ENDOIII, FPG, and 8-OHdG among healthy subjects that were oxidatively stressed by exercise or hyperbaric oxygen treatment.^{25,31}

Unlike vitamin C and vitamin E, the carotenoids have no established beneficial health effect that would define these as vitamins. In antioxidant intervention studies, carotenoids are ingested as carotenoid-rich foods (e.g., tomatoes) or model constituents (e.g., β -carotene or rutin). Supplementation with rutin (500 mg/day for 6 wk) or a mixture of α -carotene and β -carotene (15 mg/day for 12 wk) had no beneficial effect on oxidative DNA damage in terms of 8-OHdG, ENDOIII and FPG sites.^{20,32}

Multiple Supplements

Three studies have investigated the effect of combinations of antioxidants with somewhat mixed conclusions. A study that only included male smokers investigated the effect of two different formulations of vitamin C (500 mg/d) together with vitamin E (182 mg/d). Vitamin C formulated as slow-release tablets was associated with fewer ENDOIII and FPG sensitive sites after 4 wk supplementation, whereas the same dose of vitamin C, formulated as plain-release tablets, had no beneficial effect on DNA oxidation.²² Ingestion of the slow-release tablets was associated with smoother fluctuations of the plasma vitamin C concentration, and the beneficial effect may be explained by the resultant, more stable, plasma vitamin C concentrations. Ingestion of multi-vitamin tablets has also produced mixed results. Supplementation with 100 mg vitamin C, 280 mg vitamin E, and 25 mg β -carotene per day for 20 wk resulted in lower levels of ENDOIII sites among males, with the most pronounced effect among the smokers.²⁷ Another study on multi-vitamin supplementation showed no effect on 8-OHdG, detected by ELISA, among heavy smokers consuming tablets with 250 mg vitamin C/day, 200 IU α -tocopherol/day, and 6 mg β -carotene/day for 6 months.³³ It should be noted that the interpretation of the latter study might be hampered by a marked period effect that reduces the statistical power in the study. In addition, 8-OHdG was measured by an antibody-based ELISA detection system; this assay may be a useful tool for visualization of damage in cellular DNA, but it is probably not a reliably quantitative assay.³⁴

Table 1. Multiple administration of dietary antioxidants with assessment of oxidative DNA damage in white blood cells

Supplement per Day	Subjects ^a	Age (Yr) ^b	Method	Effect on Antioxidants ^c	Effect	Ref.
Vitamin C (80, 200, or 400 mg) or placebo for 15 wk followed by a 10 wk washout period	154 MF (NS)	18-64	HPLC	No	Unaltered 8-OHdG	30
Vitamin E (1000 IU) or placebo for 12 wk prior to an exercise test (downhill run)	32 M (NS)	18-35 and 65-80	HPLC	Yes	Lower levels of 8-OHdG among young subjects following supplementation, and no effect among the old. No effect following exercise.	25
Vitamin E (800 mg) or no supplementation for 7 d prior to hyperbaric oxygen treatment	6 MF (NS)	20-39	Comet	NR	No effect on ENDOIII and FPG sites	31
Subjects receiving either 5 or 15% PUFA diet supplemented with vitamin E (80 mg) or not (5-7 mg) for 4 wk	21 M (NS)	29 ± 1	Comet	Yes	Low vitamin E group: decreased ENDOIII (15% PUFA), increased ENDOIII (5% PUFA). High vitamin E group: no effect on ENDOIII sites	26
α/β-carotene (15 mg) or placebo for 12 wk	40 MF (NS)	25-45	Comet, HPLC	Yes	No effect on ENDOIII, FPG sites, and 8-OHdG	32
Rutin (500 mg) or placebo for 6 wk	16 F (NR)	18-48	Comet	Yes	No effect on ENDOIII (marked period effect)	20
Vitamin C (500 mg as slow or plain release) and vitamin E (182 mg), or placebo for 4 wk	48 M (S)	39 ± 12	Comet	Yes	Decreased ENDOIII and FPG sites by slow release tablets, but no effect from plain release tablets	22
Multivitamin tablet (100 mg vitamin C, 280 mg vitamin E, and 25 mg β-carotene) for 20 wk	100 M (50S)	50-59	Comet	Yes	Decreased ENDOIII sites after 20 weeks	27
Multi-vitamin (250 vitamin C, 200 IU α-tocopherol, and 6 mg β-carotene) or placebo for 6 mo	63 MF (S)	42 ± 9	Antibody	Yes	No difference in 8-OHdG between placebo and supplemented groups, but a decline in both groups during the trial.	33
Soy milk, rice milk, or cow milk (1L) for 4 wk	10 M (NS)	20-50	Comet	Yes	Decreased ENDOIII sites for soy milk.	28
Rye crisp bread (76.5 g) or placebo (fiber-free bread) for 2 wk	12 F (NR)	NR	Comet	No effect	No effect on ENDOIII sites	35
Flavonol (quercetin) rich diet for 2 wk in crossover design among type 2 diabetes patients	10 MF (3S)	60 ± 7	Comet	Yes	No effect on ENDOIII sites.	36

continued on next page

Table 1. Continued

Supplement per Day	Subjects ^a	Age (Yr) ^b	Method	Effect on Antioxidants ^c	Effect	Ref.
Parallel study of blackcurrant juice, anthocyanine drink or placebo (475-1000 ml) for 3 wk	57 MF (6S)	19-52	Comet	Yes	No effect on ENDOIII and FPG sites	37
Vegetable/fruit (500 g) in crossover design for 3 wk with 2 wk washout	22 M (S)	33 ± 11	Comet	Yes	No effect on ENDOIII sites	38
Vegetable/fruit (600 g), tablets with the same concentration of antioxidants/minerals, or placebo for 24 days	43 MF (NS)	27 ± 6	Comet	Yes	No effect on ENDOIII and FPG sites	42
Cruciferous and legume sprouts (113 g) or placebo for 2 wk	18 MF (NR)	21-45	Comet	No effect	No effect on FPG sites	40
Kiwi fruit (1-3 pieces) crossover for 3 wk with 2 wk washout between supplement	14 MF (NS)	26-54	Comet	Yes	Decreased ENDOIII and FPG sites, but without dose-effect relationship	29

^a Number of subjects indicated as males (M) and females (F). Smokers (S) and nonsmokers (NS) are indicated in brackets. ^b Age is shown as range or mean ± standard deviation. ^c The effect of the antioxidants in plasma or WBC may increase or decrease depending on the type of study. This effect is considered successful (yes) if the study reported statistically significant ($\alpha < 0.05$) alterations of at least one antioxidant concentration compared to baseline or placebo groups. ^d The two juices contain a mixture of apple, mango, and orange juice as basis ingredients; one juice contained aronia, blue-berries, and boysenberries (anthocyanine-rich fruits), whereas the other juice contained green tea, apricot, and lime (flavanol-rich substances).

Antioxidant Rich Foods

Eight studies on antioxidant-rich foods met the criteria to be included in the analysis. Ingestion of a diet rich in flavonols (including quercetin) and cruciferous and legume sprouts (113 g/day for 2 wk) did not alter the level of ENDOIII and FPG sites, respectively.^{36,40} Ingestion of rye crisp bread (76.5 mg/d for 2 wk) as a source of lignans was not associated with increased plasma enterolactone concentration, and had no effect on ENDOIII sites.³⁵ The null effect finding of lignans in WBC is probably reasonable considering the low bioavailability of the active substances in rye crisp bread, whereas the effects in the gastrointestinal tract are easier to comprehend. Drinking blackcurrant juice or anthocyanine drinks (475-1000 ml/day for 3 wk) also had no beneficial effect on ENDOIII and FPG sensitive sites; in fact there was a tendency that the level of FPG sites increased in the group of subjects drinking blackcurrant juice.³⁷ Anthocyanines have low bioavailability and the dose was rather high; it may be speculated that the subjects unintentionally suffered from slight intoxication of the gastrointestinal tract (e.g., some of the subjects in the active groups complained of nausea). Two studies investigated the effect of vegetables and fruits; a cross-over study with male smokers showed no effect on ENDOIII sites after ingesting 500 g/day for 3 wk,³⁸ whereas a placebo-controlled parallel study of 600 g/day for 24 days among nonsmoking subjects of both sexes was negative with respect to ENDOIII and FPG sites.⁴² The quality of these studies is high and the null effects are quite robust. Drinking soy milk (1000 ml/day for 4 wk) as a source of phytoestrogens increased plasma levels of genistein and daidzein, but not enterolactone; assessment of DNA damage revealed lower levels of ENDOIII.²⁸ The only study showing consistent effect by more than one endpoint is a study of kiwi fruit supplementation (1-3 kiwi fruits/day for 3 wk), which showed lower levels of ENDOIII and FPG sensitive sites.²⁹

An overall summary of the studies shows that six investigations reported a beneficial effect of antioxidant supplementation, whereas 12 studies reported a null effect. Studies usually set the significance level at 5%; this implies that one out of 20 studies should show a statistical significance by chance even if there is no biological effect. This means that the distribution observed here indicates that there is an over-representation of studies showing a beneficial effect of dietary antioxidants in terms of oxidative DNA damage in WBC. However, it is difficult to explain why some studies show beneficial effects and other null findings. We regard this as weak support for the notion that ingestion of antioxidant-rich foods is associated with lower spontaneous level of oxidative DNA damage in WBC than single antioxidants. On the other hand, there is virtually no evidence suggesting that high intake of antioxidants or antioxidant-rich products are associated with adverse effects in terms of increased generation of oxidative DNA damage. Although this is reassuring, it is not a proof of a more-is-better notion because there is an insufficient number of high-quality studies to address this firmly in the high-dose range.

Effect of Antioxidant Supplementation on 8-OHdG Levels in Urine

Measurement of urinary excretion of 8-OHdG in antioxidant intervention studies is based on the notion that it decreases following a steady state ingestion of antioxidants, because of a decreased rate of generation of oxidatively damaged DNA in the body. However, the effect may remain unaltered if the DNA repair system is upregulated in studies lasting less than 24h. Excretion of 8-OHdG is thought to be the result of sanitisation of the nucleotide pool by MTH1 directed pathways and possibly so far unknown endonuclease and nucleotide excision repair pathways,^{43,44} whereas 8-OHGua is derived from excision of the lesions in the DNA by the 8-oxo-guanine glycosylase (OGG1) and other glycosylases. This is illustrated in OGG1 deficient mice that have unaltered excretion of 8-OHdG, whereas 8-OHGua is excreted in lesser amounts compared to repair-proficient mice, although the difference is only by 25% suggesting alternative base excision repair.⁴⁵ So far, antioxidant intervention studies have mainly been performed with 8-OHdG as a urinary biomarker, whereas 8-OHGua and many other oxidized DNA bases in urine would also be interesting to study.

Table 2 outlines the characteristics of 25 studies with controlled design of the effect of antioxidant supplementation on urinary excretion of 8-OHdG.^{20,42,46-69,71} Four studies on single carotenoid supplementation showed no effect on urinary excretion of 8-OHdG,^{20,46,52,63} whereas a mixture of carotenoids (daily intake: α -carotene (1.4 mg), β -carotene (6.0 mg), lycopene (4.5 mg), bixin (11.7 mg), lutein (4.4 mg), and paprika carotenoids (2.2 mg) revealed a statistically significant difference in delta values (i.e., the difference between data obtained at the end of the supplementation and baseline) between the active and placebo groups, but there was no difference relative to the baseline values.⁵⁶ In fact, the statistically significant difference of the delta values was mainly caused by an increased 8-OHdG excretion in the placebo group and the excretion only decreased slightly in the group receiving mixed carotenoids. Supplementation with single or combination of vitamin C and vitamin E had no effect in most studies,^{46,47,51,55,67} whereas one study reported a beneficial effect of high dose supplementation with vitamin C (1000 mg/day) and vitamin E (600 mg/day) in HIV-infected patients who had been treated with zidovudine.⁶⁵ Multi-vitamin tablet supplementation provided no beneficial effect in normal subjects,^{42,58,59} subjects at high altitude⁶⁸ and subjects undergoing cold-weather field training at moderate altitude.^{49,54}

Investigations of natural food products show an equal distribution between the studies reporting beneficial and null effect. Ingestion of olive oils with high content of phenolic compounds was associated with reduced urinary excretion of 8-OHdG.⁴⁸ A number of studies supplied antioxidants as berries, fruits, tea, and vegetables. Ingestion of capsules containing extracts of fruits and berries, and eating diets rich in carotenoids lowered the excretion of 8-OHdG.⁵⁹ A third, but small, study that did not report changes in plasma antioxidant concentration, showed lower 8-OHdG concentration in the urine of subjects, enrolled in a soccer summer training camp, who drank a commercial vegetable juice.⁶⁰ On the other hand, neither eating 600 g of fruit and vegetables, nor the corresponding amount of minerals and vitamins in tablet form, were associated with lower urinary excretion of 8-OHdG relative to the placebo group, whereas there was a pronounced decline in the urinary excretion of 8-OHdG during the study in all the groups (i.e., a period effect).⁴² Ingestion of capsules containing juices and powder of fruits (apple, orange, pineapple, papaya, cranberry, and peach) and vegetables (carrot, parsley, beet, broccoli, kale, cabbage, spinach, and tomato) had no effect on 8-OHdG excretion in urine.⁶⁶

Supplementation with Brussels sprouts (300 g/day) was investigated in two studies with mixed outcome.^{61,64} The first study that included male subjects, concluded that Brussels sprouts lowered urinary excretion of 8-OHdG.⁶⁴ In the subsequent study of sexes, the effect was less clear; there was a tendency that only the males benefited from ingestion of Brussels sprouts, but the results were not firm because of the low number of subjects and one of the male subjects had unrealistically high urinary 8-OHdG excretion.⁶¹ Three studies have investigated the effect of drinking green tea.^{50,53,57,69} In one study there was a beneficial effect of drinking 300 ml/day for one week,⁵⁰ whereas there was no effect of ingesting green tea extract in meat patties for 3 wk.⁵³ The unadjusted data of the third study did not indicate a beneficial effect of drinking green tea; however adjustment of the data for a number of variables, including baseline 8-OHdG levels, revealed a statistically significant effect of drinking green tea for 4 months.^{57,69} Lastly, drinking soya hypocotyl tea was associated with lower urinary excretion of 8-OHdG.⁶² It should be emphasized that the fate of the antioxidants in these investigations was inconclusive because (1) the plasma concentration of carotenoids decreased; (2) the putative active constituents (isoflavones) were not measured in the plasma and the alterations in the urinary concentration could not be assessed due to insufficient information.⁶²

An overall assessment of the antioxidant intervention studies on urinary excretion of 8-OHdG do not outline overt differences between studies reporting beneficial and null findings related to the duration of the intervention period, number of subjects, or the power to detect a 50% difference. There was an equal number of studies reporting beneficial and null effects that investigated 8-OHdG in urine by HPLC and ELISA. The collection period of urine differed

Table 2. Urinary excretion of 8-OHdG in antioxidant supplementation studies

Supplement per Day	Duration	Subjects ^a	Age ^b	Baseline DNA Damage	Assay (Collection Period)	Effect on Antioxidants ^c	Effect	Ref.
Rutin (500 mg) β-carotene (30 mg)	6 wk	16 F (NR)	18-48	86.3 ng/mg creatinine	ELISA (spot)	Yes	No effect	20
	1 mo	14 M (NS)	19-22	1.8 mmol/mmol creatinine	HPLC (24 h)	Yes	No effect before or after an exercise test	52
β-carotene (20 mg) Carotenoids ^d	14 wk	122 M (S)	39	NR	HPLC (24 h)	Yes	No effect	63
	3 wk	32 MF (NS)	32 ± 11	21.6 ng/mg creatinine	ELISA (spot)	Yes	No effect versus baseline, but decreased 8-oxodG in active group	56
Vitamin C (500 mg)	3 wk	30 MF (NS)	17-49	9.3 pmol/μmol creatinine	ELISA (spot)	Yes	post-supplementation No effect due to vitamin C, 47,71 but increased excretion in washout period	67
Vitamin C (500 mg) to patients with systemic lupus erythematosus Six groups receiving combinations of vitamins ^e	6 wk	15 F (NS)	24-75	9.0 pmol/μmol creatinine	ELISA (spot)	Yes	No effect	46
	2 mo	116 M (S)	30-65	42.4 nmol	HPLC (24 h)	Yes	No effect	65
Vitamin C (1 g) and vitamin E (0.6 g)	1 mo	13 M (NR)	30 ± 3	NR	HPLC (24 h)	NR	Lower 8-OHdG in the active group of HIV-infected patients receiving zidovudine therapy	51
2x2 parallel study of vitamin C (500 mg) and vitamin E (400 IU)	2 mo	184 MF (NS)	58 ± 14	17.8 ng/mg creatinine	ELISA (24 h)	Yes	No effect	

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Table 2. Continued

Supplement per Day	Duration	Subjects ^a	Age ^b	Baseline DNA Damage	Assay (Collection Period)	Effect on Antioxidants ^c	Effect	Ref.
2x2 parallel study of vitamin C (500 mg) and vitamin E (182 mg)	12 mo	48 M (22S)	45-69	0.42 nmol/kg bw	HPLC (24 h)	Yes	No effect	55
Multivitamin tablet ^f	2 wk	30 NR (NR)	22 ± 1	157 ng/l per kg fat-free mass	ELISA (24 h)	Yes	No effect (subjects undergoing cold-weather training at moderate altitude)	49
Multivitamin tablet ^g	21 d	39 MF (NR)	7 ± 2	11.8 ng/mg creatinine	ELISA (spot)	Yes	No effect	58
Multivitamin tablet ^h	24 d	40 M (NR)	18-40	7.5 ng/mg creatinine	ELISA (overnight)	Yes	No effect (subjects undergoing cold-weather training at moderate altitude)	54
Multivitamin tablet ⁱ	35 d	18 M (NS)	26 ± 5	3.4 ng/mg creatinine	HPLC (24 h)	Yes	No effect	68
Fruit and vegetable capsules ^j	7 wk	59 MF (11S)	50 ± 6	5.1 mmol/mol creatinine	ELISA (spot)	Yes	No effect	66
Fruit and vegetable (600 g), tablets with the same concentration of antioxidants/minerals, or placebo	24 d	43 MF (NS)	27 ± 6	19.3 nmol	HPLC (24 h)	Yes	No effect, but a decline in all groups	42
Fruit juice (480 ml) ^k	4 d	11 M (NR)	21 ± 1	340.9 ng/kg bw	ELISA (12 h)	NR	Decreased 8-OHdG in active group	60
Brussel sprouts (300 g)	1 wk	10 MF (NS)	NR	625.4 pmol/kg bw	HPLC (24 h)	NR	No effect	61
Brussel sprouts (300 g)	12 d	10 M (NS)	NR	494.8 pmol/kg bw	HPLC (24 h)	NR	Decreased 8-OHdG in the active group	64

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Table 2. Continued

Supplement per Day	Duration	Subjects ^a	Age ^b	Baseline DNA Damage	Assay (Collection Period)	Effect on Antioxidants ^c	Effect	Ref.
Antioxidant rich diet or tablets ¹	5 wk	55 MF (NR)	71 ± 6	5.4 mmol/mol creatinine	ELISA (24 h)	Yes	No effect versus baseline, but decreased 8-OHdG in active group	59
Green tea, black tea, or water (4 cups)	1-4 mo	120 MF (S)	18-79	9.7 ng/mg creatinine	ELISA (spot)	Yes	Decreased 8-OHdG after 4 mo in green tea group, but not before	57,69
Green tea (300 ml)	7 d	40 M (S)	18-24	NR	HPLC (12 h)	NR	Decreased 8-OHdG	50
Green tea (32 oz)	7 d	28 MF (12S)	25-45	NR	HPLC (12 h)	NR	Decreased 8-OHdG	50
Green tea extract ^m	3 wk	16 M (8S)	20-31	337 pmol/kg bw	HPLC (24 h)	Yes	No effect related to supplementation but a decreased excretion during the study in all groups	53
Soya-topoty ^l tea (>1 l)	1 mo	38 F (NR)	NR	NR	ELISA (NR)	Altered ⁿ	Decreased 8-OHdG in active group (statistical test not reported)	62
Polyphenol-rich olive oils (25 ml)	40 d	12 M (NS)	20-22	12.1 nmol/mmol creatinine	HPLC (spot)	Yes	Decreased following supplementation in a dose-dependent manner	48

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Table 2. Continued

^a Number of subjects indicated as males (M) and females (F). Smokers (S) and nonsmokers (NS) are indicated in brackets. ^b Age are shown as range or mean $\pm$ standard deviation. ^c The effect of the antioxidants in plasma of WBC may increase or decrease depending on the type of study. This effect was regarded as successful (yes) if the study reported statistically significant ($\alpha < 0.05$) alterations at least one antioxidant concentration compared to baseline or placebo groups. ^d Supplement constitute β -carotene (6 mg), α -carotene (1.4 mg), lycopene (4.5 mg), bixin (11.7 mg), lutein (4.4 mg), paprika carotenoids (2.2 mg). ^e The six groups had daily supplementations with 1) 200 mg vitamin E; 2) 500 mg plain-release vitamin C; 3) 500 mg slow-release vitamin C; 4) 90 mg coenzyme Q10 in oil; 5) 30 mg coenzyme Q10 granulate; 6) placebo. ^f Tablets contain β -carotene (12 mg), vitamin E (400 IU), vitamin C (500 mg), selenium (100 mg), and zinc (30 mg). ^g Tablets contain micronutrients similar to 3 fruits and 3 vegetables (including 107 mg vitamin C and 83 mg vitamin E). ^h Consisted of β -carotene (20050 IU), vitamin C (330 mg), tocopherols (650 IU), selenium (167 mg), catechin (13.2 mg), lutein (500 mg), lycopene (100 mg), N-acetyl-1-cysteine (181 mg), pomegranate extract (5 mg). ⁱ Consisted of β -carotene (20000 IU), α -tocopherol (400 IU), vitamin C (500 mg), selenium (100 mg), and zinc (30 mg). ^j The fruit capsules were made from juiced apple, orange, pineapple, papaya, cranberry and peach, and the vegetable capsules were from carrots, parsley, beet, broccoli, kale, cabbage, spinach, and tomato. The daily dose consisted of 200 mg vitamin C, 60 mg vitamin E, and 15 mg β -carotene. ^k Contain vitamin A (240 mg), β -carotene (399mg), vitamin C (219 mg), vitamin E (1.44 mg) per day. ^l Consisted of tablets with vitamin antioxidants (400 mg vitamin C, 150 mg vitamin E, 4 mg β -carotene), capsules (90 mg vitamin C, 18 IU vitamin E, 2.4 mg β -carotene, and powder or extract of fruits and berries), or a carotenoid-rich diet. ^m Consisted of 1000 mg extract/kg bodyweight in meat patties (total phenolics were 23.5 mg/10 MJ). ⁿ Carotenoids decreased in both the control and active groups relative to baseline, but there was no difference between plasma levels at 4 wk of supplementation. Plasma isoflavone concentrations were not reported and the results of urinary excretion of isoflavones were too limited to provide statistical analysis.

between studies with either spot samples or 24 h collections as the preferred choices; two studies used overnight urine and 12 h collections.^{54,60} We cannot discern any difference between the two detection methods with respect to producing studies showing protective or null effects of antioxidant supplementations. A stratification of the studies, according to the administrations as single, multiple, or antioxidant-rich foods, show that studies reporting a beneficial effect have provided more complex antioxidant supplements than studies reporting null effect (0/2/7 and 6/6/4 for single, multiple, and antioxidant-rich foods in studies reporting beneficial and null effect, respectively; $\chi^2 = 6.7$, $p < 0.05$).

Conclusions

There are numerous experimental studies published each year on the potential of antioxidants or antioxidant-rich foods, to prevent of oxidation of DNA. On an experimental basis it is easy to understand that ingestion of antioxidants should be associated with lower levels of DNA oxidation. However, many of the studies have limited value because of methodological problems related to the study design and assays, and lack the power to detect 50% differences between two groups. Although single studies may have higher power due to specific designs such as repeated measurements, it is a concern that many studies have too few subjects. The most likely effect ratio in healthy subjects is less than 10% difference, which means that the number of subjects should be in the hundreds, rather than tens.

A firm conclusion from the antioxidant intervention studies cannot be reached. From the data, there is a trend to support the idea that supplementation with antioxidant-rich food can decrease the urinary excretion of 8-OHdG, whereas single antioxidants do not. In WBC there is support for the notion that long-term antioxidant supplementation lowers the basal level of oxidative DNA damage. The number of single dosing antioxidant trials has been too few to generate firm conclusions on the effect of supplementation on the level of oxidative DNA damage in WBC. Collectively, these findings can be interpreted as an overall beneficial effect in the whole body, but the use of WBC as a surrogate tissue may not be particularly well suited for the detection of this effect. It should also be kept in mind that the majority of the studies include healthy individuals and it is possible that the protective effect of antioxidants is more easily detected in subjects who are under oxidative stress. This could be healthy subjects exposed to oxidative stress (e.g., exhaustive exercise and hyperbaric oxygen treatment) or patients with oxidative stress related diseases. The few, well-controlled, studies that reported realistic levels of oxidative DNA damage in WBC of oxidatively stressed subjects, do not lend too much support to the notion that such a population would benefit from antioxidant supplementation compared to a healthy, normal study population. However, it should also be recognized that most of these studies used supplementation with vitamin E, anticipated to be the least effective type of antioxidant, with respect to DNA oxidation. There is an obvious requirement for more, well controlled antioxidant intervention studies, encompassing putatively oxidatively stressed subjects with oxidative DNA damage measured by enzymic or chromatographic methods.

In the future, more attention should be addressed on alternative chemopreventive mechanisms, such as up-regulation of DNA repair systems, as well as and other types of DNA damage, e.g., bulky DNA adducts. Supplementation with kiwi fruits or cooked carrots appears to be associated with increased DNA repair activity.^{29,70} Also the chemopreventive effect of antioxidants in nonlymphatic tissue is sparsely investigated, but should be addressed thoroughly before important beneficial effects of antioxidants are discounted. Very few studies have investigated dose-response relationships, including doses with possible toxic effects, of antioxidants or antioxidant-rich foods. Thorough investigations of the dose-response relationships should be considered in future studies on both antioxidant effects and other chemopreventive mechanisms. Hopefully these future studies will benefit from the lessons learned from the antioxidant intervention studies, namely that proper design and focus on the validity of the biomarkers are pivotal determinants for the interpretations of the studies.

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